HL156 compounds, novel metformin derivatives, inhibit angiogenesis through AMPK mediated downregulation of FOXM1 in gastric cancer cells

Hae-Ji Choi

Department of Medical Science

The Graduate School, Yonsei University

HL156 compounds, novel metformin derivatives, inhibit angiogenesis through AMPK mediated downregulation of FOXM1 in gastric cancer cells

Directed by Professor Jae-Ho Cheong

The Master`s Thesis

Submitted to the Department of Medical Science

the Graduate School of Yonsei University

in partial fulfillment of the requirements for the degree of

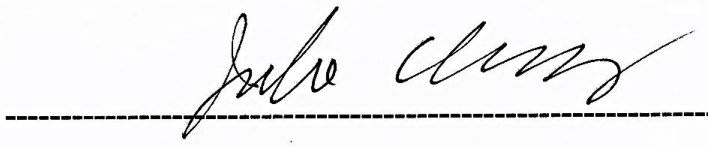
Master of Medical Science

Hae-Ji Choi

December 2014

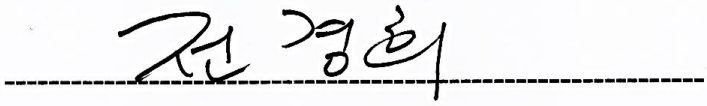
This certifies that the Master`s Thesis

of Hae-Ji Choi is approved.



Thesis Supervisor: Jae-Ho Cheong

Thesis Committee Member #1: Ho-Geun Yoon



Thesis Committee Member #2: Kyung-Hee Chun

The Graduate School

Yonsei University

December 2014

ACKOWLEDGEMENTS

짧으면서도 길게 느껴졌던 2년 동안의 여정의 성과물인 학위 논문을 완성 할 수 있게 되었습니다. 혼자 힘으로는 절대 이루어 낼 수 없었을 것입니다. 2012년부터 처음 실험실 생활을 시작하면서 많은 분들의 도움으로 지금까지 올 수 있었던 것 같습니다.

먼저, 우리 지도 교수님, 정재호 교수님께 감사의 마음을 전합니다. 아무것도 모르던 저에게 아낌없는 가르침과 관심 덕분에 제가 여기까지 올 수 있었던 것 같습니다. 아직 부족하지만 교수님을 알게 되고 교수님께 배울 수 있게 되어 정말 영광이고 감사합니다.

학위 과정 동안 자문과 조언을 해주신 윤호근 교수님과 전경희 교수님 두 분께 감사의 인사를 드립니다.

처음 만났던 때가 지금도 생생하게 기억나는 우리 실험실 사람들 박은성 교수님, 박기청 박사님, 기현정 박사님, 정민 언니, 이재은 박사님, 심문희 선생님, 이도형 선생님, 박경호 선생님, 최윤영 선생님 그리고 정말 많이 믿고 의지하고 같이 있어서 힘들어도 둘이어서 웃으며 이겨낼 수 있었던 아라 모두 감사합니다.

항상 잊지 않고 반갑게 맞이해주는 성락 오빠, 한슬 언니, 은정 언니, 선영 언니 그리고 이곳에서의 소중한 인연들인 지혜, 지은, 인수 오빠, 성호 오빠, 주만 오빠, 동국 오빠 모두에게 감사합니다.

각자의 길을 열심히 가고 있는 오랜 친구들 수진, 혜영, 진희, 연진, 가영, 금진, 세나 모두에게 고마움을 전합니다.

마지막으로 저를 믿고 묵묵히 뒤에서 지켜봐 주시는 우리 가족 아빠, 엄마, 오빠 감사하고 사랑합니다.

최 혜 지

TABLE OF CONTENTS

Ⅲ. RESULTS

LIST OF FIGURES

ABSTRACT

HL156 compounds, a novel metformin derivative, inhibit angiogenesis through AMPK mediated downregulation of FOXM1 in gastric cancer cells

Hae-Ji Choi

Department of Medical Science

The graduate School, Yonsei University

(Directed by Professor Jae-Ho Cheong)

Metformin, which is widely used as an anti-diabetic drug, has recently gained significant attention as a potential anti-cancer agent. Although a couple of potential mechanisms explaining anti-tumor effects have been suggested, its detailed molecular mechanisms remain elusive. HL156 compounds are novel biguanide derivatives based on metformin formula with more potent anti-tumor effects than

metformin. In cell based assays, HL156 activates AMP-activated kinase (AMPK) more potently than metformin in various cancer cell lines.

Our preliminary analysis of Reverse phase protein array (RPPA) data from gastric cancer patients indicated that proteins related to regulating angiogenesis were increased in tumors of patients with poor prognosis. In addition, a pilot *in vivo* efficacy study showed that HL156 significantly suppressed metastasis in breast cancer xenograft models.

Motivated by these, we hypothesized that HL156 would inhibit angiogenesis that is one of the critical steps of metastatic process. Further, the anti-angiogenic mechanism of HL156 would be mediated through AMPK activation which downregulated oncogenic transcription factor FOXM1.

Culturing HUVEC cells in the conditioned media treated with HL156 compounds to gastric cancer cells downregulated *in vitro* endothelial cell network formation. In the same conditioned media, the expression of VEGFA, a major angiogenic factor, was prominently suppressed. Also, the expression of FOXM1, a pleiotropic oncogenic transcription factor, was inhibited by HL156, which is correlated with activation of AMPK. To further interrogate the molecular mechanisms by which FOXM1 was down-regulated, we assessed CDK4/6 expression in gastric cancer cells after HL 156 treatment. CDK4/6, which phosphorylates and activates FOXM1, was downregulated leading to the decrease of phospho-FOXM1 (Ser35) level. Electrophoretic mobility shift assay showed that HL156 treatment repressed the FOXM1 binding to the VEGFA promoter.

Taken together, these results suggest that HL156 alleviates tumor angiogenesis by VEGFA inhibition through AMPK mediated CDK4/6 - FOXM1 downregulation in gastric cancer cells.

Key words: HL156, angiogenesis , gastric cancer, ampk, foxm1, cdk4/6

HL156 compounds, a novel metformin derivative, inhibit angiogenesis through AMPK mediated downregulation of FOXM1 in gastric cancer cells

Hae-Ji Choi

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Jae-Ho Cheong)

Ⅰ. INTRODUCTION

The biguanide metformin (1,1-dimethylbiguanide hydrochloride) is a widely used oral hypoglycemic agent for treatment of type 2 diabetes[1]. During the decades, epidemiologic, preclinical and clinical studies suggested the associations of diabetes and metformin with cancer. Metformin has recently gained significant attention as a potential anti-cancer agent that inhibits the proliferation of several human cancer cell lines including prostate [2], breast [3], colon [4], glial [5], and gastric cancer [6].

AMP-activated protein kinase (AMPK) is a sensor of energy status that maintains cellular energy homeostasis by fine-tuning a range of diverse cellular processes. In mammals, there are seven genes

encoding AMPK, two isoforms of α (α1 and α2), two of β, (β1 and β2), and three of γ (γ1, γ2 and γ3). The α subunits contain a typical serine/threonine kinase domain at the N terminus; as for many protein kinase, these only having significant activity when phosphorylated by upstream kinases at conserved threonine residue within the activation loop (Thr172 in human α1)[7] .

Anti-cancer effect of metformin is known to be mainly mediated through the activation of LKB1/AMPK pathway [8]. AMPK activation results in down-regulation of mammalian TOR complex1 and the IGF-1/AKT pathways, and in p53-mediated cell-cycle arrest [9]. Active AMPK leads to loss of cyclin D1 mRNA and protein and the decline in cyclin D1 levels causes the release of sequestered CDK inhibitors, $p27^{Kip1}$ and $p21^{Cip1,}$ which then bind to and inhibit the cyclin E/CDK2 complex [10].

Of note, metformin suppresses mitochondrial electron transport chain complex Ⅰ [11], which is now recognized as a major effect of metformin mediated tumor suppression. When combined with 2-deoxyglucose (2DG), metformin induces cancer cell death *in vitro*, significantly suppressed tumor growth in two distinct xenograft tumor models *in vivo* [12]. Further, metformin decreases angiogenesis by increasing the anti-angiogenic thrombospondin-1 (TSP-1) in insulin resistant obese women[13]. Recently metformin demonstrated the anti-angiogenic effect through the suppression of VEGF expression, mediated by downregulation of the mTORC1/HIF-1α pathway [14].

HL156 compounds, novel metformin derivatives with enhanced AMPK activating property, have proved tumor growth inhibition and anti-metastasis effect in breast cancer cell *in vivo* models at low concentrations. (Pilot study 1,2)

Tumor metastasis is a multistage process during which malignant cells spread from the primary site to distant organs. The process of cancer metastasis consists of sequential and interrelated steps. Each of these can be rate limiting, because a failure or an insufficiency at any steps can halt the process [15, 16].

Angiogenesis is a new blood vessel formation from pre-existing ones in the essential process of metastatic cascade. Extensive vascularization must be required for a tumor mass exceeding diameter of 1-2mm [17]. The synthesis and secretion of angiogenic factors has a critical role in establishing a capillary network within the surrounding host tissues [18]. Local invasion of the host stroma by tumor cells occurs

by multiple mechanisms [16]. Thin-walled venules and lymphatic channels, both of which offer very little resistance to penetration by tumor cells [19]. After tumor cells have survived in the circulation, they become arrested in capillary beds of distant organs by adhering to vessel walls [20]. Extravasation occurs next, by mechanisms similar to those that involved during the initial vascular invasion. Proliferation of the tumor cells within the organ parenchyma completes the metastatic process. The micrometastasis must establish vascularization [21] and defenses against host immune system to colonize and enter these processes for additional metastases [19, 20].

Expression of the metastatic signature has been correlated with a poor prognosis and has been shown to be consistent in several types of cancer. A growing tumor needs an extensive network of capillaries for provision of nutrients and oxygen as well as for disposal of metabolic wastes [22]. Therefore, tumor growth and metastasis are angiogenesis-dependent.

To initiate the formation of new capillaries, endothelial cells (ECs) of pre-existing blood vessels must degrade the underlying basement membrane and invade into the stroma of the neighbouring tissue by the activity of the plasminogen activator (PA) system and the MMPs (matrix metalloproteinases) [23]. After proteolytic degradation of the ECM (extracellular matrix), endothelial cells migrate to the degraded matrix by a variety of growth factors including VEGF family [24], FGF-2 [25] and angiopoietins [26]. The final phases of the angiogenic process, including the construction of capillary loops and the determination of the polarity of the endothelial cells, are mediated by cell adhesion molecules [27].

VEGF/VEGF-receptor (VEGFR) axis plays a central role in angiogenesis. VEGF has been associated with tumor progression and poor prognosis in colorectal carcinoma [28, 29], gastric carcinoma [30, 31], pancreatic carcinoma [32, 33], breast cancer [34, 35], prostate cancer [36], lung cancer [37] and melanoma [38].

The mammalian VEGF family consists of five glycoproteins including VEGFA, VEGFB, VEGFC, VEGFD and placenta growth factor (PIGF) [24, 39]. The most important molecule that controls blood-vessel morphogenesis is VEGFA. VEGFA is required for the chemotaxis and differentiation of endothelial precursor cells (EPCs), EC proliferation, the direct assembly of ECs into vasculogenesis

and angiogenic remodelling [40]. Under hypoxic conditions, VEGF transcription is upregulated by hypoxia-inducible factors (HIF) [41, 42].

Targeting the VEGF pro-angiogenic signalling pathway with various inhibitors has led to FDA approval of angiogenesis inhibitors as cancer therapeutics [43]. A ligand-trapping monoclonal antibody bevacizumab (Avastin, Genetech/Roche), and two kinase inhibitors (sorafenib (Nexavar, Bayer) and sunitinib (Sutent, Pfizer)) targeting the VEGF receptor (VEGFR) tyrosine kinases are clinically tested for the proof-of-concept for anti-angiogenesis therapy [44]. However, inherent or acquired resistance to VEGF pathway inhibitors can occur in most patients, leading in some cases to a lack of response and in others to disease recurrence [45].

VEGF effects on cell survival have been shown to be mediated by Flk1/VEGFR2-PI3K-Akt pathway [46, 47]. Akt is a critical regulator of PI3K-mediated cell survival [48, 49]. In many tumors, AKT is overexpressed or amplified with elevated level of AKT phosphorylation [50, 51].

PI3K/AKT signaling pathway also plays an important role in regulating the vasculature and angiogenesis[52, 53]. PI3K/AKT regulates tumor angiogenesis by down-stream targets such as mTOR/p70S6K1 signaling axis, the inhibition of FOXO, the induction of NO [54, 55], and the inhibition of GSK-3β.

The forkhead box O (FoxO) transcription factors FOXO1 and FOXO3A are critical regulators of endothelial sprout formation and migration *in vitro* [56]. Inhibition of Foxo1 activity in mature endothelial cells, has been reported to be an important mechanism through which angiopoietin 1 (Ang1) modulates endothelial function [57], and inhibition of FOXO factors promotes endothelial proliferation [58]. In addition, FOXO3A represses the proximal VEGF promoter through FOXM1 dependent and independent mechanisms in breast cancer [59]. Forkhead box protein M1 (FOXM1) belongs to the forkhead superfamily of transcription factors which are identified by an evolutionary conserved Forkhead/winged-helix DNA-binding domain [60, 61]. It is previously known as Trident (in mouse) [62], HFH-11(in human) [63], WIN or INS-1(in rat) [64], MPP-2 (partial human cDNA) [65] or FKHL-16 [66]. The

human FOXM1gene is a 10-exon structure spanning approximately 25 kb on the 12p13-3 chromosomal band [62].

The FOXM1 transcription factors are crucial for G1-S and G2-M cell cycle phase progression and mitotic spindle integrity [67]. FOXM1 regulates a variety of biological processes in mammalian cells through regulating the transcription of genes important for cell cycle progression, cell proliferation, survival, cell differentiation, DNA damage repair, angiogenesis, cell migration, and chemotherapeutic drug response [68]. FOXM1 expression is stimulated by oncogenes and growth factors and inhibited by p53. Rb and p19Arf inhibit activity of FOXM1. A new study showed that FOXM1 could stimulate expression of genes involved in various steps of tumor metastasis [69], including epithelial-mesenchymal transition (EMT), cell migration, and premetastatic niche formation.

The FOXM1 protein exists in three different isoforms. Both FOXM1B and C splice variants are transcriptionally active, whereas FOXM1A is transcriptionally inactive, due to the disruption of the transactivation domain in FOXM1A [63]. FOXM1 is a proliferation specific mammalian forkhead transcription factor: its expression is restricted to cells that are proliferating [61]. The FOXM1 gene exhibits a specific expression pattern during the cell cycle. Both FOXM1 mRNA and protein levels increase at the entry to the S-phase of the cell cycle and remain elevated during the G2 and M-phases [62]. The transcriptional activity of FOXM1 is also cell cycle-restricted, and depends on its phosphorylation level, reaching maximum levels in the G2/M transition. Based on the putative consensus phosphorylation sites, kinases such as CDK2, CDK1, mitogen-activated protein kinase (MAPK) and Plk-1 may be involved in FOXM1 phosphorylation. FOXM1C is strongly activated by cyclin D1/CDK4 and antagonistically regulated by cyclin D1/CDK4 and RB [70]. CDK4/6 initiate FOXM1 phosphorylation, by that means resulting accumulation of FOXM1 in cells, thereby activate and stabilize FOXM1 by direct multisite phosphorylation [71]. Cyclin-CDK-dependent phosphorylation of the C-terminal region of FOXM1 increases transcriptional activation causing recruit a transcriptional co-activator, the histone deacetylase p300/CREB binding protein (p300/CBP). FOXM1 also interacts with CDK inhibitor $p27^{kip}$ which inhibits Cyclin-CDK kinase [61]. MAPK-mediated phosphorylation of FOXM1 was shown to regulate subcellular localization and transcriptional activation[72]. FOXM1

possesses 5 RXL (cyclin binding) motifs and 15 CKD consensus sites, 12 of which are localized in its C-terminal transactivation domain (TAD)[71].

Cyclin-dependent kinases (CDKs) govern cell cycle phase transitions in mammals and boost global gene transcription. Cyclin D-dependent kinases (CDK4 and CDK6) have been identified as the major oncogenic drivers among cell cycle CDKs. At the mechanistic level, recent studies have revealed pro-tumorigenic functions of CDK4/6 beyond cell cycle progression. Cells possess three distinct families of regulatory subunits: activating D-type cyclins, negative regulatory INK4 proteins and Cips/Kips, which can act as both inhibitors and activators, depending of their phosphorylation status [73].

Gastric cancer is the second most leading cause of cancer-related death worldwide [21], and the overall prognosis of advanced gastric cancer is poor [74]. In gastric cancer, expression of AKT and phosphorylated AKT (p-AKT) are detected in 74% and 78% of tumors respectively [75]. In a report of 50 advanced gastric carcinomas, there was a statistically significant correlation between p-AKT expression and depth of tumor, number of involved lymph nodes and poor prognosis [76]. Compared with other more extensively investigated cancers, such as breast, prostate, and colon carcinoma, the molecular mechanisms involved in the transformation and progression of gastric cancer are poorly characterized [77].

Our preliminary analysis of Reverse phase protein array (RPPA) data from gastric cancer patients indicated that p-AKT, PAI1, VEGFR2 and p-FOXO3A all of which related with angiogenesis were increased in tumors of patients with poor prognosis. Together with a pilot study that demonstrated anti-metastasis effect of HL156 in *in vivo* breast tumor mouse models, we were prompted to test the hypothesis that HL156 inhibits angiogenesis. In this study, we demonstrated that HL156 compounds suppress VEGFA expression in gastric cancer cell line models. Further, we found that the downregulation of oncogenic transcription factor FOXM1, which transcriptionally activates VEGFA expression, is mediated through inhibition of cyclin D-dependent kinase, CDK4/6 by AMPK activation.

Ⅱ. MATERIALS AND METHODS

1. Reagents and Cell treatment

Cells were cultured in 10-cm dishes containing 10ml of the medium. After attachment for 24hours and growth 80% confluence, the cells were treated with 30μM HL156 compounds. A stock solution of 25mM HL156 compound was dissolved and diluted with distilled water to obtain final concentrations of 30μM. Compound C (Merck KGaA, Darmstadt, Germany), the AMPK inhibitor, was stocked at a concentration of 20mM (dissolved in DMSO) and was diluted with DMSO for the final doses of 20μM (0.1% DMSO). Control cells were treated with an equal amount of distilled water or DMSO.

2. Cell lines and culture conditions

MKN28 and AGS cells were maintained in RPMI-1640 medium containing 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, and 100 ug/ml streptomycin. Human umbilical cord endothelial cells (HUVECs) were maintained in EGM-2 Bulletkit (contains FBS, Hydrocortisone, hFGF-B, GA-1000, hEGF, Heparin, VEGF, R^3-IGF-1, ascorbic acid) purchased from Lonza (Walkersville, MD USA). Cells were grown at 37℃, 5% CO_2 humidified incubator.

3. Western blotting

Protein lysates were prepared from cells at 90% confluency. The cells were then washed in ice-cold phosphate buffered saline (PBS) and then extracted in a lysis buffer containing 1% Triton X-100, 50mmol/L HEPES (pH7.5), 150mmol/L NaCl, 25mmol/L β-glycerophosphate, 25 mmol/L NaF, 5mmol/L EGTA, 1mmol/L EDTA as a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The lysates were centrifuged at 13,000 rpm for 30min and frozen at -80℃ until use. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Samples were electrophoresed using 8% SDS-PAGE, and proteins were transferred to PVDF membranes. The membranes were incubated with primary antibodies after blocking and then were incubated with HRP-conjugated secondary antibodies. Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system on X-ray film. In this study, the following antibodies were used : anti-β-actin monoclonal antibody (Sigma-Aldrich;A5441, used at 1:1000), FOXM1 (Cell signaling, #5436, used at 1:1000), FOXO3A (Cell signaling, #2497, used at 1:1000), FOXO1 (Cell signaling, #2880, used at 1:1000), Phospho-FOXM1(Ser35) (Cell signaling, #14170, used at 1:1000), cyclin D1(Cell signaling, #2926, uses at 1:1000), CDK4 (Cell signaling, #12790, used at 1:1000), CDK6 (Cell signaling, #3136, used at 1:1000), p27 (Santa cruz, sc-528, used at 1:1000), Phospho-AMPKα (Thr172) (Cell signaling, #2535, used at 1:1000), AMPKα (Cell signaling, #2532, used at 1:1000), HIF1α (millipore, #07-628, used at 1:1000).

4. MTS cell proliferation assay

Cell proliferation assays were conducted using the MTS assay (Promega Corporation, Madison, WI, USA) according to the manufacturer`s instructions. Each cell line ($1X10^4$) was seeded into a well of a 96-well plate and cultured in 100㎕ of RPMI-1640 supplemented with 10% FBS.

After 24hours, seeding cells were treated with 10nM, 100nM, 1μM, 100μM, 500μM, 1mM HL156 compound or, as a control, without HL156 compound. After treatment, 20㎕ MTS[(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)], was added to each well and the cells were incubated at 37℃ with 5% CO_2 for 4 hours. Absorbance was measured for each well at a wavelength of 490nm using an ELISA reader.

5. Matrigel morphogenesis assay

150㎕ of growth factor reduced BD Matrigel matrix basement membrane (BD Biosciences, San Jose, CA, USA) was pipetted into each well of a 24-well plate and polymerized for 30 min at 37℃. Human umbilical vein endothelial cells (HUVECs) were harvested after trypsin treatment and suspended in conditioned medium. Next, $2X10^4$ HUVECs in 500㎕ of conditioned medium were added to each well and incubated at 37℃ in 5% CO^2 for 4hours. We then photographed the cultures under a bright-field microscope. The quantified results were expressed in number of master segments and meshes.

6. Assessment of secreted VEGFA

MKN28 and AGS cells were treated with 30μM HL156 compounds for 48hours. Supernatant medium was collected and VEGFA concentrations were measured using commercially available sandwich ELISA kits (Thermo scientific, Waltham, MA, USA).

7. Nuclear protein extraction

Nuclear and cytoplasmic extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Thermo scientific, Waltham, MA, USA). For adherent cells, harvest with trypsin-EDTA and then centrifuge at 500 X g for 5 minutes. Wash cells by suspending the cell pellet with PBS. Transfer 1-10X10^6 cells to a 1.5ml microcentrifuge tube and pellet by centrifugation at 500Xg for 2-3 minutes. Use a pipette to carefully remove and discard the supernatant, leaving the cell pellet as dry as possible. Add ice-cold CER Ⅰ to the cell pellet. Proceed to Cytoplasmic and Nuclear Protein Extraction, using the regent volumes indicated.

8. Electrophoretic mobility shift assay (EMSA) and super-shift assay

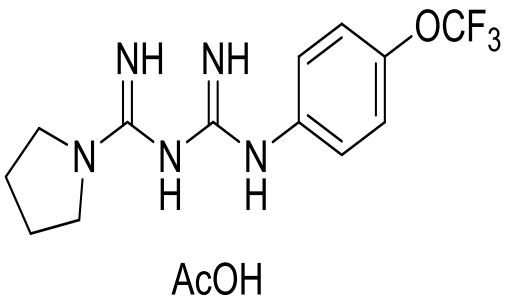
The DNA-protein binding detection kit (Promega, Madison, WI, USA) was used with modifications. In brief, DNA-protein binding reactions were carried out in a final volume of 25 μl of buffer containing 10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% (w/v) glycerol, 0.1 mg/ml sonicated salmon sperm DNA, 15 μg of nuclear extract, and oligonucleotides. Oligonucleotides containing FOXM1 binding consensus sequence were end-labeled to a specific activity of 5 x 10^5 CPM with γ-[32P]-ATP and T4-polynucleotide kinase, followed by purification on a Nick column (GE Healthcare, Piscataway, NJ). Reaction mixtures with radio-labeled oligonucleotides were incubated at room temperature for 20 minutes, and resolved on 6.5% non-denaturing polyacrylamide gels after addition of 3 μl bromophenol blue (0.1%). Gels were dried and subjected to autoradiography. For super-shift assays, 3 μg of antibody was added for 20 minutes at room temperature after the initial incubation. Abs specific for FOXM1 were purchased from Cell Signaling Technology.

9. Statistical analysis

Hierarchical cluster analysis was done using Cluster and Treeview. The reproducibility and correlation of results were tested by calculating Pearson Correlation Coefficients. The associations between protein feature and mutation status within cell line clusters were determined by x^2 and Fisher`s exact testing. Analysis of statistical associations between drug sensitivity and RPPA clusters were done using one-way ANOVA. All statistical analyses were done in the R language environment.

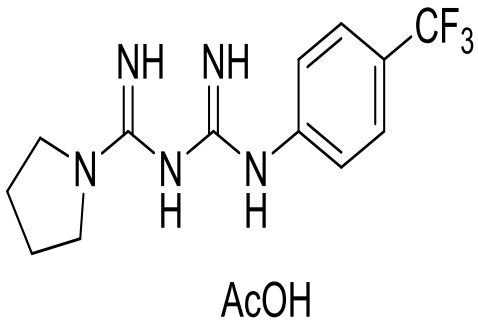
Ⅲ. RESULTS

HL156271 (HL156A)



N-(N-(4-(trifluoromethoxy)phenyl)carbamimidoyl)pyrrolidine-1-carboximidamide acetate

HL156277 (HL156B)



N-(N-(4-(trifluoromethyl)phenyl)carbamimidoyl)pyrrolidine-1-carboximidamide acetate

Figure 1. HL156 compound structure formula

1. Effect of HL156 compounds on cell proliferation

Our pilot study showed that new potential metformin derivative HL156 (Fig. 1) inhibits tumor proliferation *in vivo*. Xenograft tumor model was established by injecting human colorectal carcinoma HCT116 cells in nude mice. HL156 showed comparable tumor growth inhibition effect at lower dose (50mpk) compared to capecitabine (Xeloda®) (100mpk) (Pilot study). Based on this result, we wanted to evaluate the effect of the growth inhibition activity of HL156 on human gastric cancer cells. We examined the effect of HL156 on proliferation in two gastric cancer cell lines, MKN28 and AGS. Cells were grown in 10% FBS and treated 10nM, 100nM, 1μM, 100μM, 500μM, 1mM HL156 compound or, as a control, without HL156. The cell proliferation assay was conducted 3 days after the addition of the compound by MTS assay. HL156 slightly decreased cell proliferation in a dose-dependent manner in MKN28 and AGS cells. Based on this result, we determined 30μM for working concentration of HL156 to interrogate the biological effects of HL 156 on tumor cells at the concentration that does not induce cellular toxicity (Fig. 2).

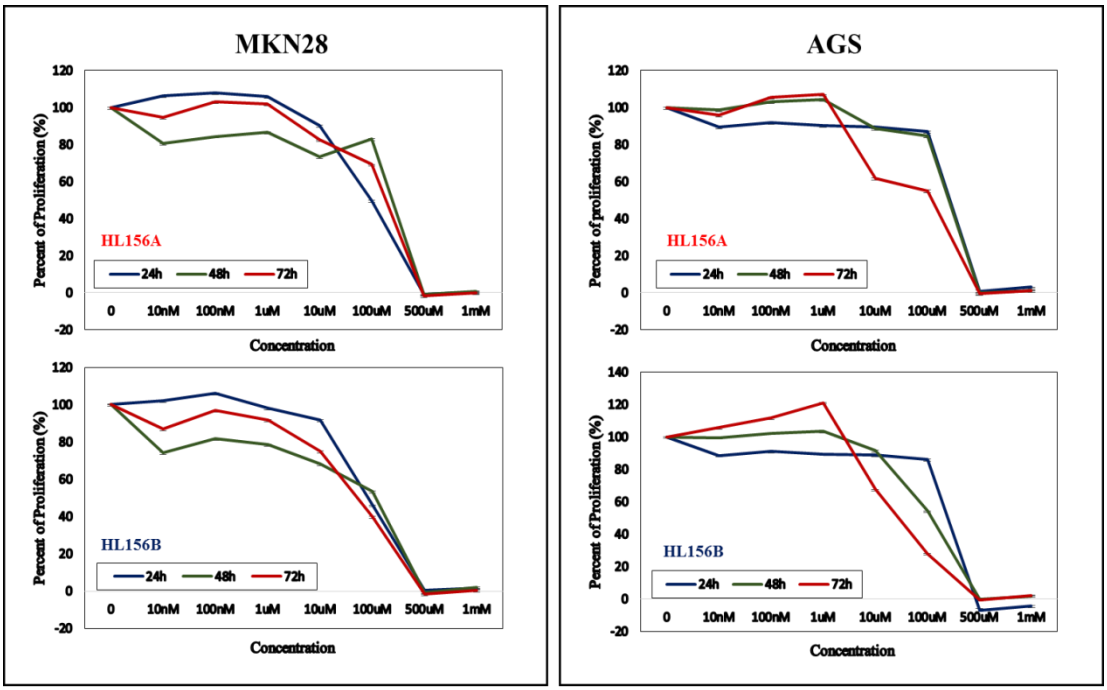


Figure 2. 30μM HL156 compounds do not significantly affect proliferation of gastric cancer cells.

AGS and MKN28 were seeded in 96-well plates. After 24 hours, HL156 compounds were added to the culture medium and viability assay was conducted daily from 0 to 72 hours. MTS assay was conducted as described in Materials and Methods. The data points represent the mean cell number from 3 independent experiments and error bars represent standard deviation.

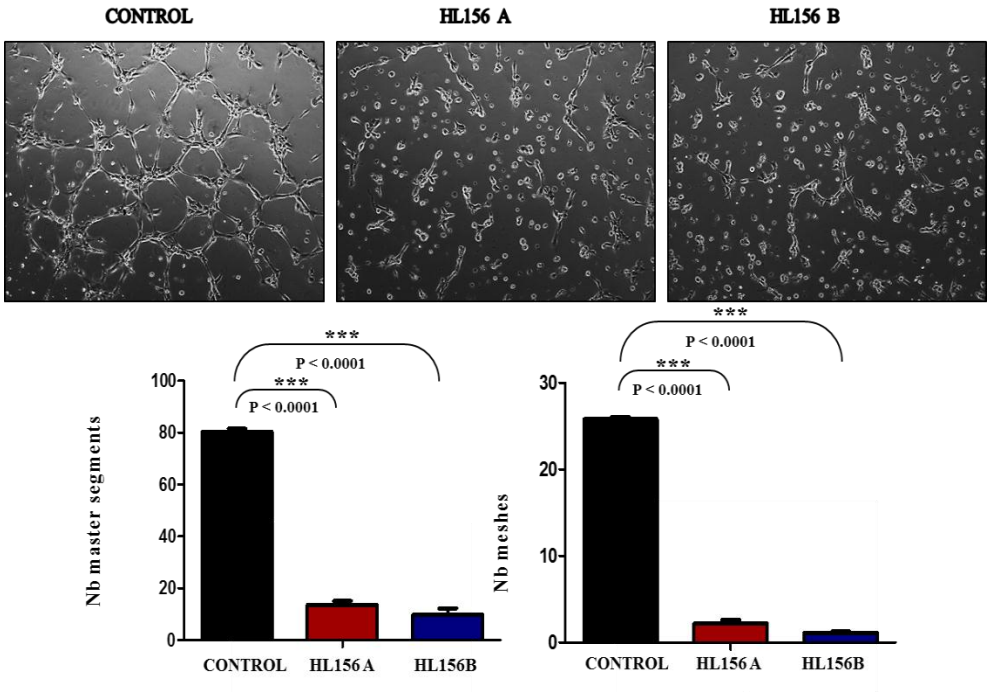
2. HL156 compounds down-regulate endothelial cell network formation

Metformin has been reported to inhibit the proliferation of several cancer cell lines[2-5] . However, there are few studies reporting effects on angiogenesis by metformin[13, 78, 79].

In our pilot study, HL156 showed anti-metastasis effect in MDA-MB-231 breast cancer mouse models. Based on these, we hypothesized that HL156 would inhibit angiogenesis which is one of the critical steps of metastatic process.

We evaluated this idea using an endothelial cell tube formation assay. Endothelial cells cultured in the conditioned media from MKN28 or AGS cells treated with 30μM HL156 for 48hours had a significantly reduced capillary tube formation compared with the conditioned media from cells treated with vehicle alone as shown by the decreases in the number of segments and meshes (Fig. 3). Taken together, these results suggested that the decreased *in vitro* capillary tube formation by HL156 might be mediated by humoral factors secreted from cancer cells.

AGS


CONTROL
HL156 A
HL156 B
Nb master segments
***
P < 0.0001
***
P < 0.0001
100
80
60
40
20
0
CONTROL
HL156 A
HL156B
Nb meshes
***
P < 0.0001
***
P < 0.0001
30
20
10
0
CONTROL
HL156 A
HL156B

MKN28

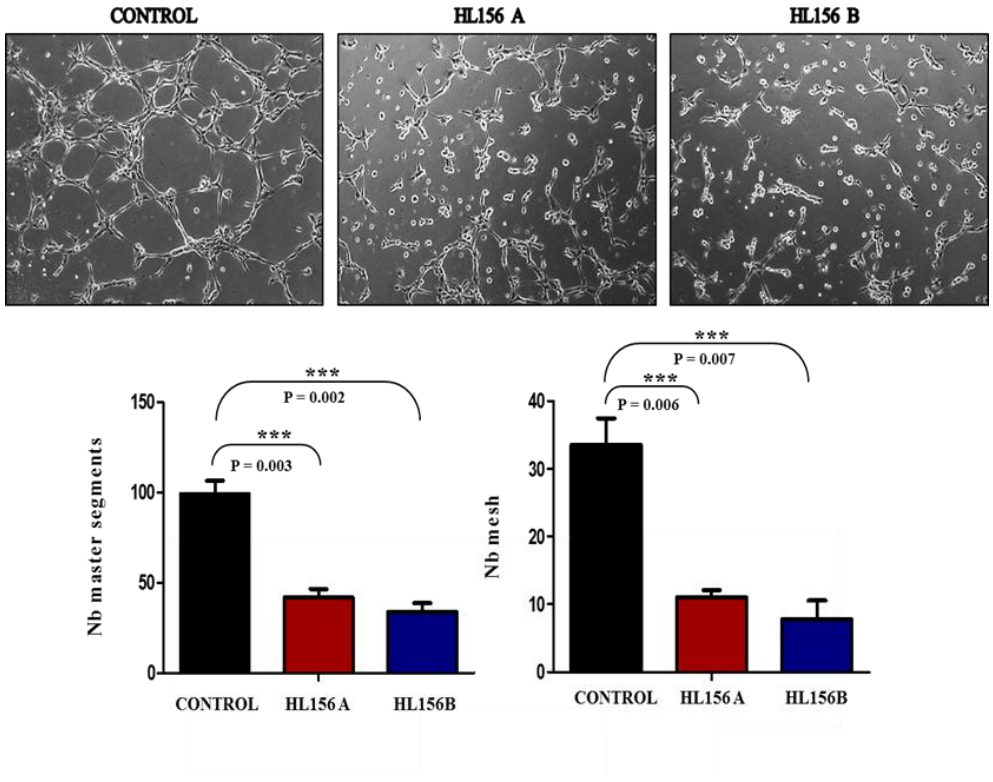

CONTROL
HL156 A
HL156 B
Nb master segments
***
P = 0.002
***
P = 0.003
150
100
50
0
CONTROL
HL156 A
HL156B
Nb mesh
***
P = 0.007
***
P = 0.006
40
30
20
10
0
CONTROL
HL156 A
HL156B

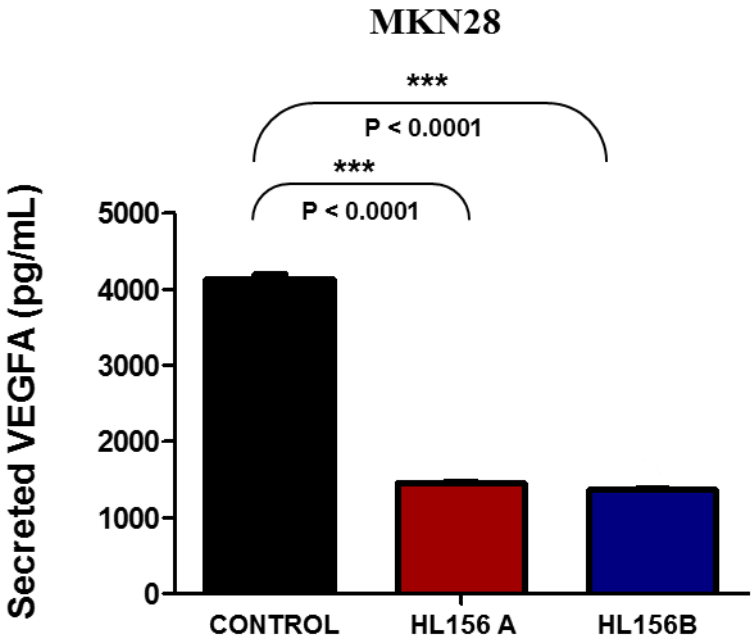
Figure 3. HL156 compounds inhibit endothelial cell morphogenesis.

The angiogenic potential of gastric cancer cells treated with HL156 was determined by an endothelial cell matrigel tube formation assay. Samples of conditioned media were prepared from MKN28 and AGS cells. Human umbilical cord endothelial cells (7×10^4) in 500 $\mu\ell$ of conditioned media were then plated on growth factor-reduced Matrigel to form a capillary tube. Data from three independent tube formation experiments. Master segments and meshes were quantified by Angiogenesis Analyzer for ImageJ software. Bar graph shows the mean and standard deviation of the measured Master segments and meshes (n=3). Statistical significance ($P < 0.05$) in a comparison of HL156 treated and control groups.

3. Regulation of VEGFA secretion by HL156 compounds in gastric cancer cells

Based on the above results, we next determined the expression of VEGFA, a secreted major angiogenic protein, in the conditioned media with or without HL156 treatment. Conditioned media from untreated MKN28 and AGS cells showed high levels of secreted VEGFA protein. On the other hand, treatment of HL156 on cancer cells decreased VEGFA levels in the conditioned media (Fig. 4A). In concordance with ELISA results, VEGFA protein levels were decreased by HL156 in AGS cells (Fig. 4B).

A.



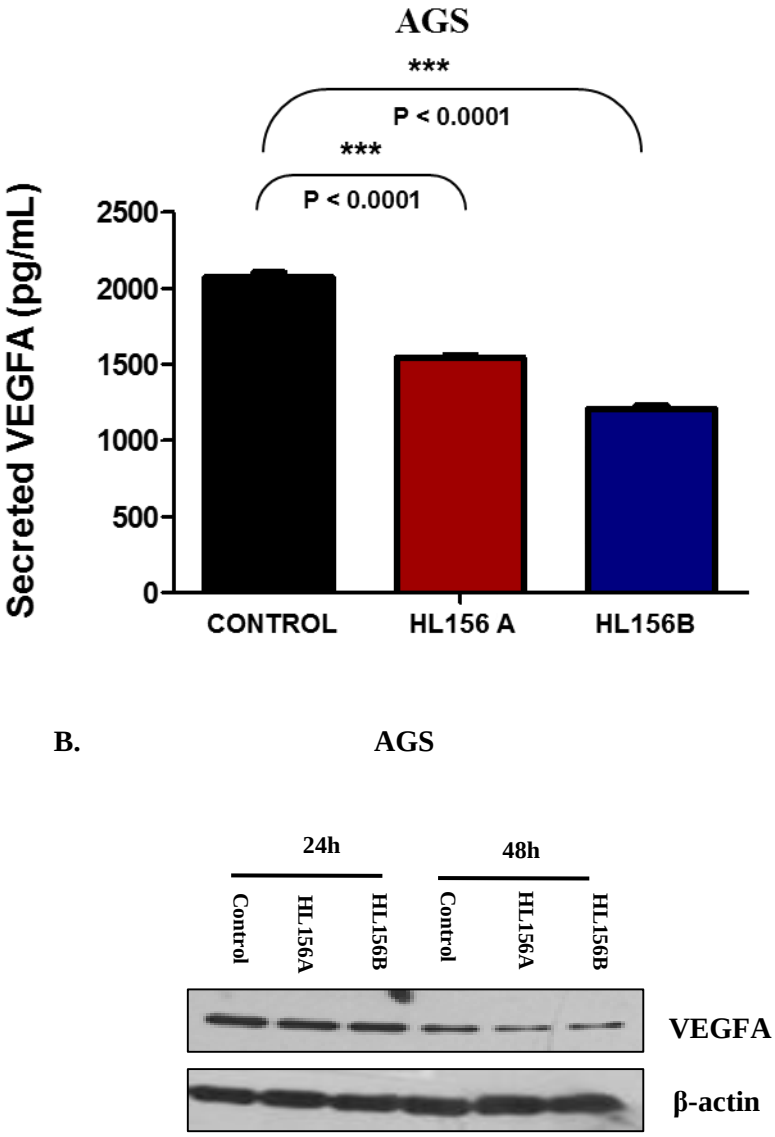


Figure 4. HL156 compounds inhibit VEGFA secretion in gastric cancer cells

A. VEGFA concentrations in conditioned media of HL156 treated gastric cancer cells were measured by a quantitative sandwich enzyme immunoassay according to the manufacturer`s protocol (Human VEGFA ELISA kit, Thermo scientific). The optical density was measured at 450nm and 550nm using an ELISA plate reader. **B.** In parallel, VEGFA protein levels of these HL156 compounds treated AGS cells were also analyzed by western blotting. β-actin as a loading control.

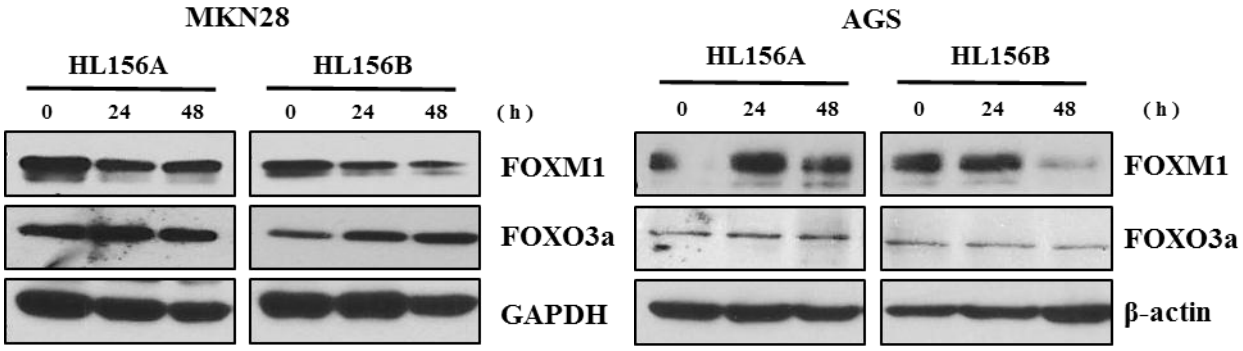
4. HL156 represses expression of FOXM1, but not FOXO3A

HIF-1α is one of the characteristics of solid tumors that promote the production of VEGF and angiogenesis[80]. Therefore, we investigated the expression levels of HIF-1α, but there are no significant changes in HIF-1α after HL156 treatment (supplementary Fig.3), indicating the effect of HL156 on VEGFA expression might be independent of HIF-1α.

Our preliminary analysis of Reverse phase protein expression array (RPPA) data from gastric cancer patients indicated that p-AKT, PAI1, VEGFR2 and p-FOXO3A were increased in tumors of patients with poor prognosis. Since AKT, PAI1 and VEGFR2 are well-established angiogenesis related proteins, we speculated that FOXO3A would also regulate angiogenesis in gastric cancer. FOXO3A, one of FOXO transcription factors, is a critical regulator of endothelial sprout formation and migration *in vitro*. In addition, FOXO3A represses the proximal VEGFA promoter through FOXM1 - dependent and - independent mechanisms in breast cancer[59]. Overexpression of FOXM1 promotes cell-cycle progression, angiogenesis, invasion and metastasis[81, 82]. In contrast, loss of FOXO3A activity may increase resistance to apoptosis and cell cycle progression[67].

Thus, we speculated that the anti-angiogenic mechanism of HL156 would be regulated by FOX protein, especially FOXO3A and FOXM1 which have antagonistic role in angiogenesis. We first monitored the expression of FOXO3A and FOXM1 upon HL156 treatment of gastric cancer cells. Western blot analysis showed that HL156 treatment of MKN28 and AGS cells caused a decrease in FOXM1, but had little effects on FOXO3A (Figure. 5A). In other gastric cancer cell lines including MKN45, NCI-N87, SNU638 the same results have obtained (Supplementary Fig. 2). Having a similar endothelial sprout formation function like FOXO3A, FOXO1, the other forkhead family member, [56] also showed few changes (Supplementary Fig. 3). In sharp contrast, nuclear FOXM1 was eminently downregulated by HL156 treatment, profoundly by HL156 B in particular, but no change in nuclear FOXO3A (Fig. 5B). These results suggest that FOXM1 is specifically down-modulated among forkhead proteins by HL156.

A. Total



B. Nucleus

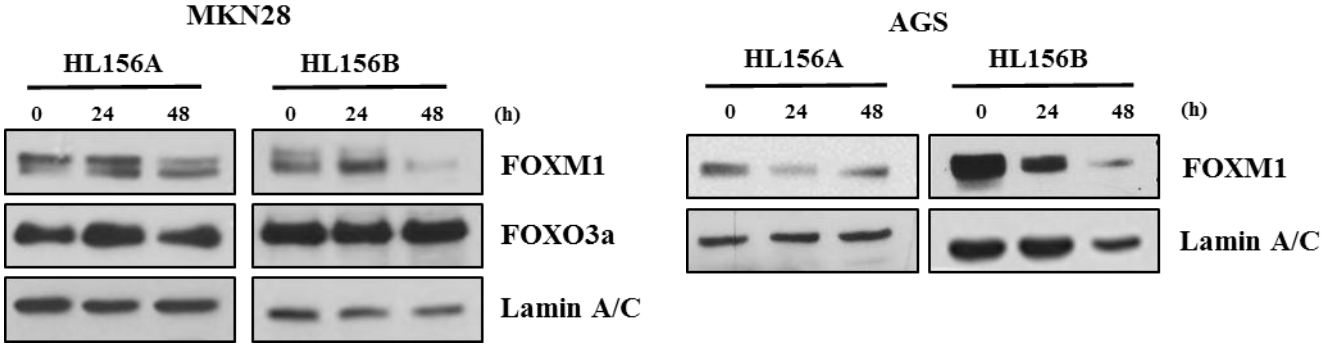


Figure 5. Expression of FOXM1 is repressed by HL156 compounds

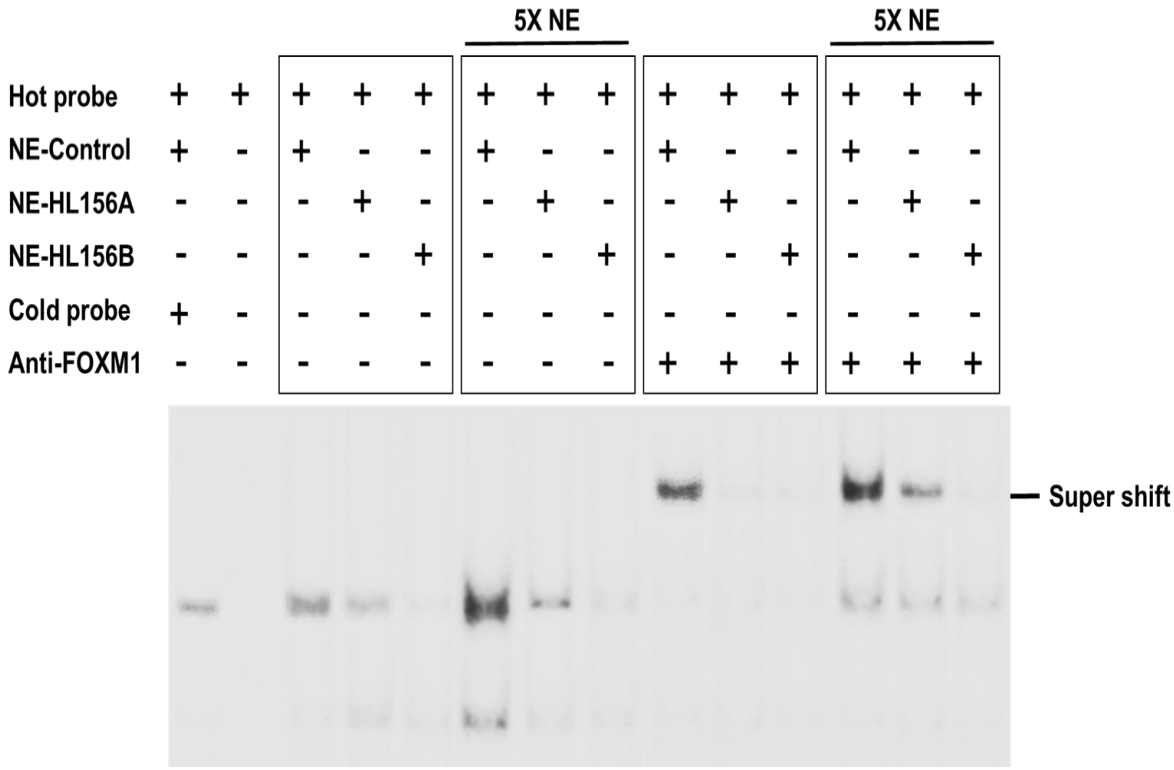
A. Total protein was isolated at 24 and 48h following HL156 treatment. Western blot analysis showed that the level of FOXM1 was reduced in gastric cancer cells (MKN28 and AGS) when treated with HL156 A/B (30μM) for indicated time points. **B.** Nuclear and cytoplasmic extracts were prepared at the times indicated, separated on polyacrylamide gels and subjected to immunoblotting with FOXM1 and Lamin A/C used for nuclear marker.

5. HL156 suppresses FOXM1 binding to the VEGFA promoter *in vitro*

Recently VEGFA was reported to be a direct transcriptional target of FOXM1[83]. To determine whether FOXM1 binding to VEGFA promoter is affected in response to HL156 treatment, we performed electrophoretic mobility shift assays using FOXM1 binding sequences 5`-TAATCA-3` [64] in VEGFA promoter.

We found that FOXM1 binding region in VEGFA promoter specifically bound to nuclear extract prepared from MKN28 cells not treated with HL156 compound resulting in the formation of major shifted band while no detectable binding was noticed in the samples treated with HL156 (Fig. 6). Furthermore, the shifted band could be supershifted by the anti-FOXM1 antibody. Together with immunoblot results in Fig. 5B, these results indicate that HL156 compounds inhibit FOXM1 expression, particularly in the nucleus, thereby impeding binding to FOXM1 specific motif in the VEGFA promoter. Of note, the intensity of shifted bands between HL156A and HL156B treatment was different proportional to protein amounts in immunoblot assay (Fig. 5B), further confirming the nuclear FOXM1 specifically binds to VEGFA promoter.

A.



B.

Figure 6. HL156 compounds suppress FOXM1 binding to the VEGFA

A. Binding of FOXM1 to the VEGFA promoter *in vitro*. We performed EMSA using nuclear protein (NE) extracted from MKN28 cells and the oligonucleotide probes of putative FOXM1 binding region of VEGFA promoter. A major shifted band was noted and was competed out by an excess of unlabeled FOXM1 binding region oligonucleotide, and supershifted by anti-FOXM1 antibody. **B.** Sequences and positions of the VEGFA promoter.

6. Increased AMPK activity by HL156 suppresses FOXM1 expression

AMPK is a key energy sensor regulating normal and cancer cell metabolism and bioenergetics homeostasis. AMP which is elevated when cellular energetics are depressed activates AMPK by promoting its phosphorylation at Thr172 and by direct activation via an allosteric AMP site [84].

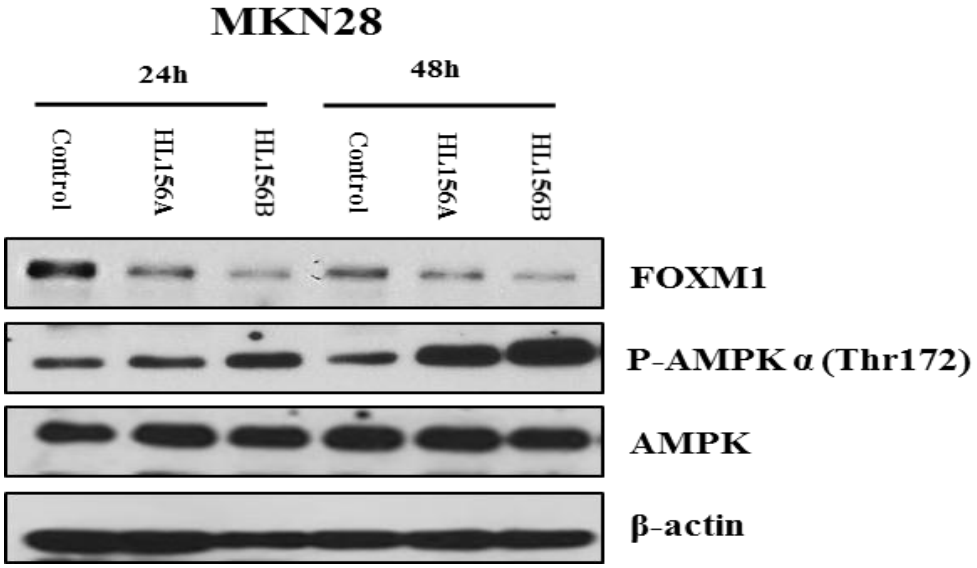
It is well known that metformin activates AMPK. Metformin is thought to activate AMPK by inhibition of Complex Ⅰ of the respiratory chain, which leads to a decrease of intracellular ATP levels [85]. AMPK has a general role in coordinating growth and metabolism according to the cellular bioenergetics level [86]. It is reported that when AMPK activity increased, the expression of FOXM1 decreased in mouse hearts after birth [87]. Further, activation of AMPK inhibits cervical cancer cell growth through reducing the expression of FOXM1 [88].

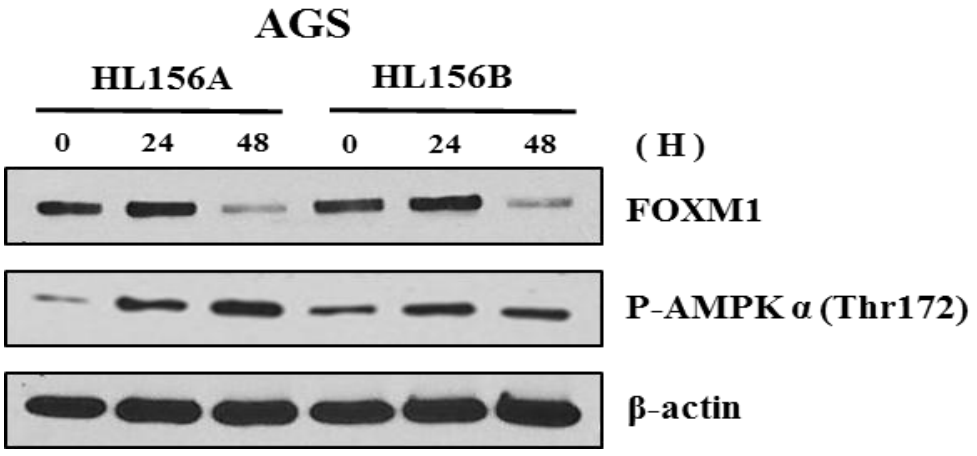
HL156 is a designed new compound with enhanced property of AMPK activation compared to metformin. It is, therefore, of interest to determine if AMPK activation in HL156 treated cells downregulates FOXM1.

The results show that HL156 promotes AMPK Thr172 phosphorylation demonstrated by using anti-phospho-AMPK antibody and that this correlates with downregulation of FOXM1 in both MKN28 and AGS cells (Fig. 7A).

To further interrogate the role of AMPK in FOXM1 downmodulation, MKN28 cells were pretreated with compound C, a potent reversible AMPK-specific inhibitor, or DMSO (vehicle) for 30 min and then treated with or without HL156 (30μM) for an additional 48 hours. Pretreatment with compound C inhibited AMPK activation and rescues the reduction of FOXM1 levels caused by HL156 (Fig. 7B). These results indicate that downregualtion of FOXM1 by HL156 is mediated through AMPK activity.

A.


MKN28
24h
48h
Control
HL156A
HL156B
Control
HL156A
HL156B
FOXM1
P-AMPK α (Thr172)
AMPK
β-actin


AGS
HL156A
HL156B
0
24
48
0
24
48
(H)
FOXM1
P-AMPK α (Thr172)
β-actin

B.

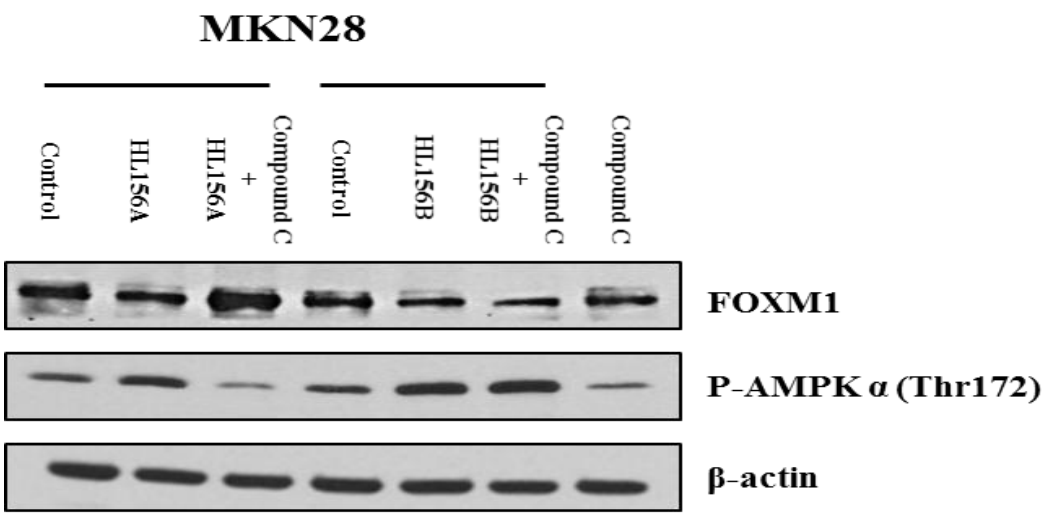


Figure 7. Activation of AMPK by HL156 compounds suppresses expression of FOXM1

A. Western blot analysis showed that the level of p-AMPKα (Thr172) was increased, while the level of FOXM1 was reduced in gastric cancer cells (MKN28, AGS) when treated with HL156 (30μM) for indicated times. **B.** Pre-treatment of AMPK inhibitor, compound C (20μM) reverses the effects of HL156 compound and recues the level of FOXM1.

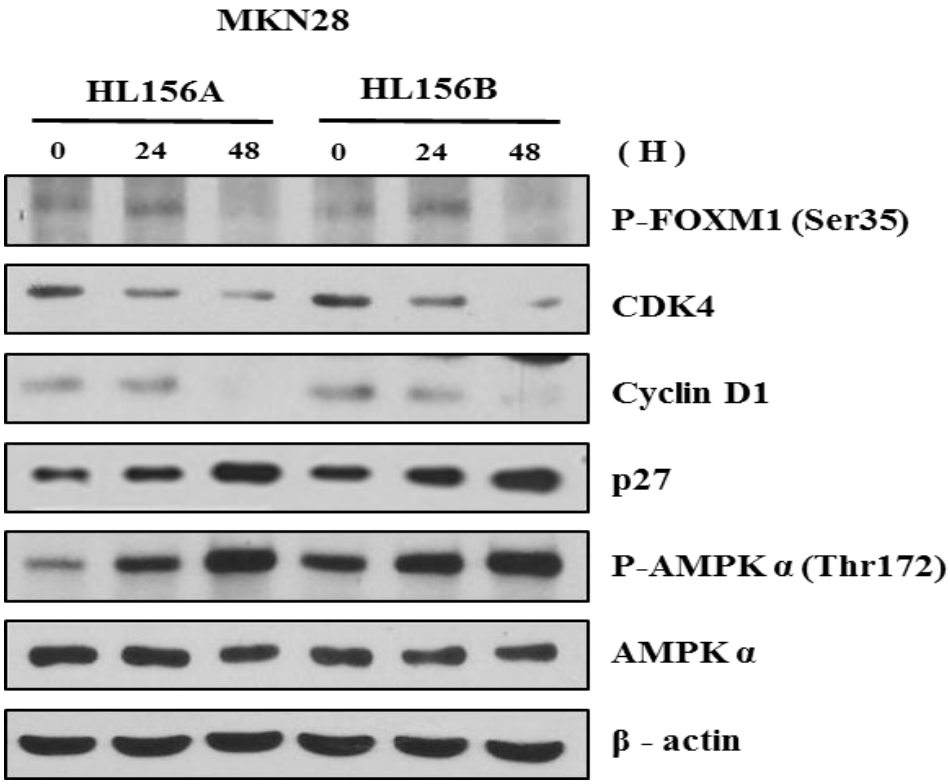
7. Effect of HL156 on cell cycle regulatory proteins which regulated phosphorylation of FOXM1

Next, we raised a question about molecular players that mediate AMPK activation and downregulation of FOXM1. The transcriptional activity of FOXM1, which follows its phosphorylation status, also increases and peaks at the G2/M transition [89]. Activation of cyclin D dependent kinase is regulated by D-type cyclins, negative regulatory INK4 proteins and Cips/Kips which can act as both inhibitors and activators [73]. Reliance of the abundance and stoichiometry of the three regulator subunits (D-cyclins, Cip/Kips, INK4) in the cell, each of the catalytic subunits (CDK4 or CDK6) can either bind to a D-type cyclin and a Cip/Kip protein, thus forming a trimeric holoenzyme [73]. Down-regulation of FOXM1 using siRNA reduced expression of cyclin D1, increased expression of CDK inhibitor $p27^{kip90}$. FOXM1 is identified that it is stabilized and activated by CDK4/6 as a multisite phosphorylation target[71]. Multisite phosphorylation of FOXM1 (Ser4, Ser35, Ser331, Ser451, Ser489, Ser508, Thr510, Ser522, Thr600, Thr611, Thr620, Thr627, Ser638, Ser672, Ser704) by cyclin D-CDK4/6 protected FOXM1 from degradation. Metformin reduces cyclin D1 [2, 10], cyclin D- dependent kinase (CDK) 4, CDK6 in human gastric cancer cell lines [6].

We hypothesized that activation of AMPK by HL156 compound inhibits CDK4/6, decreases FOXM1 phosphorylation, and reduces FOXM1 accumulation in the nucleus, thereby decreasing binding of FOXM1 to the VEGFA promoter.

The protein levels of CDK4 significantly declined 48h after the treatment of HL156 compound. On the contrary, the CDK inhibitor $p27^{kip}$ expression elevated in cells treated with HL156 (Fig. 8).

Phospho-FOXM1 (Ser35), one of the N-terminal CDK4/6 target sites, also significantly decreased, concordant with the above result. Taken together, our data suggest that increased AMPK activity down-regulates FOXM1 mediated by CDK4/6 suppression.


MKN28
HL156A
HL156B
0
24
48
0
24
48
(H)
P-FOXM1 (Ser35)
CDK4
Cyclin D1
p27
P-AMPK α (Thr172)
AMPK α
β - actin

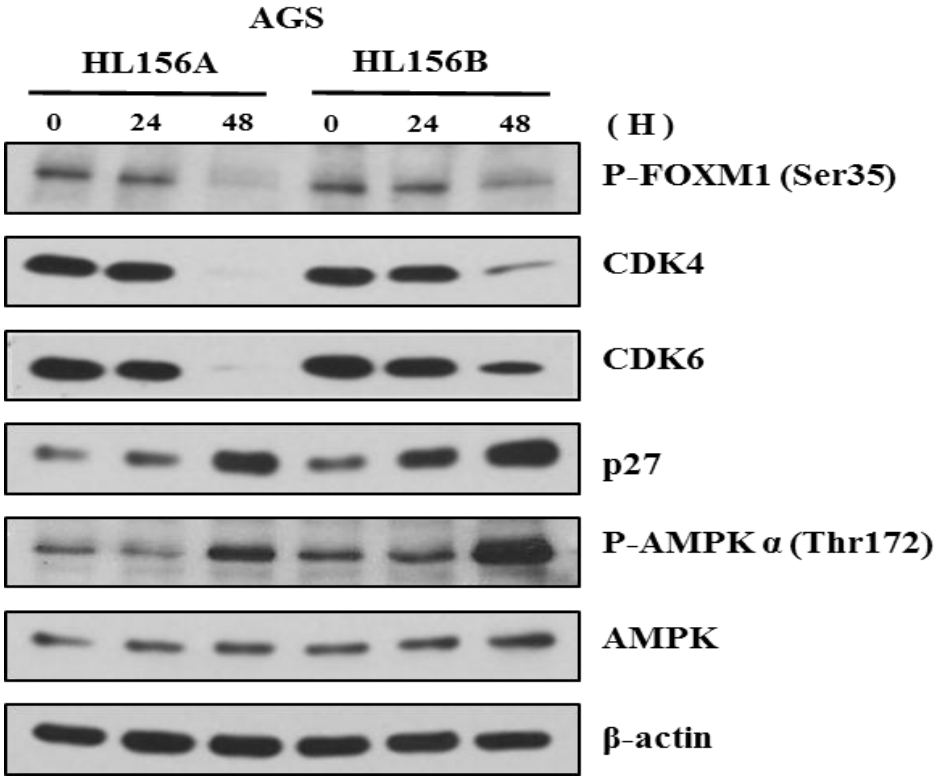

AGS
HL156A
HL156B
0
24
48
0
24
48
(H)
P-FOXM1 (Ser35)
CDK4
CDK6
p27
P-AMPK α (Thr172)
AMPK
β-actin

Figure 8. Downregulation of CDK4 corresponds to activation of AMPK

Expression of CDK4 decreased in both MKN28 and AGS cells treated with HL156 for 48 hours. Consequently, phospho - FOXM1 (Ser35), a main phosphorylation site by CDK4/6, was also decreased. Western blotting of Cyclin D1, CDK4, CDK6, and phosphorylated FOXM1 (Ser35) in both MKN28 and AGS cells of 24 and 48 hours after the addition of 30μM of HL156.

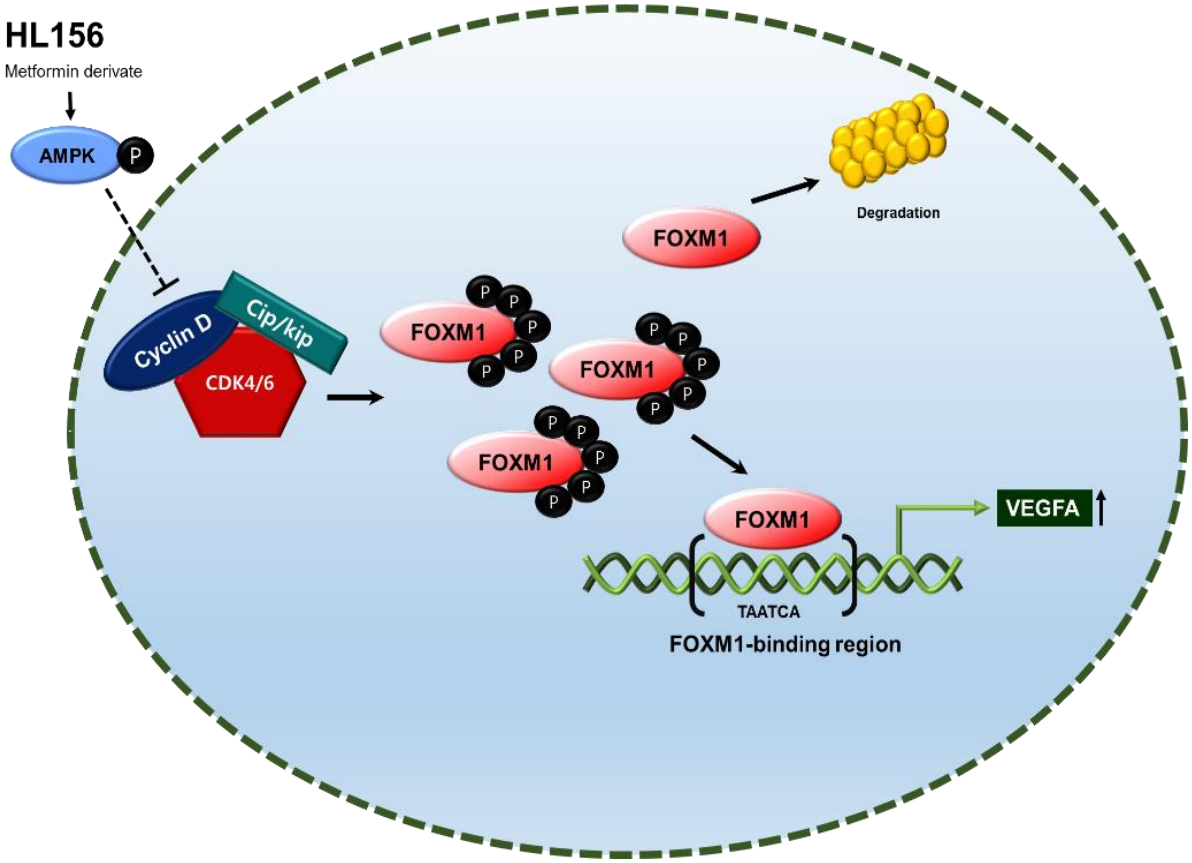


Figure 9. Proposed model for the mechanism of HL156 compounds mediated anti-angiogenesis

Activation of AMPK leads to downregulation of CDK4/6 proteins. The reduction in CDK4 affects phosphorylation level of FOXM1. As unphosphorylated FOXM1 being increased, transcriptional activity of FOXM1 decreases due to protein degradation. Thus, AMPK→CDK4/6→FOXM1 signaling blocks transcription of FOXM1 target genes, especially VEGFA.

Ⅳ. DISCUSSION

To summarize, the results presented here are consistent with a model in which increased phosphorylation and activation of AMPK by HL156 compounds leads to the downregulation of oncogenic transcription factor FOXM1, resulting in the anti-angiogenic effects.

We here discovered the anti-angiogenic effect of a novel potent metformin derivative, HL156 on VEGFA secretion in gastric cancer cells. We found that VEGFA secretions were significantly reduced by HL156. These suppressive effects were observed at 30uM, which did not significantly affect the proliferation of gastric cancer cells, a very lower concentration than that used in traditional anti-cancer studies on metformin (5-20 mM) [91]. Moreover, we found that HL156 specifically suppressed oncogenic transcription factor FOXM1 which regulates VEGFA expression through direct binding to Forkhead binding elements (FHRE) of VEGFA promoter region [68].

AMPK activation has been demonstrated a potent anti-proliferation effect in cancer [2] and we questioned whether this might be related with inhibition of angiogenesis. Downregulation of FOXM1 in response to metformin has been demonstrated in cervical cancer cells through AKT/FOXO3A signaling pathway [88]. Our data showed that the reduction of FOXM1 expression is correlated with activation of AMPK, however, the inhibition of FOXO3A by AKT is not reduced when AMPK is activated. Using compound C, a specific inhibitor of AMPK, reversed HL156 induced downregulation of FOXM1 in gastric cancer cells. HL156 B also more powerfully downregulates FOXM1 than HL156 A. It remains to be determined appropriate Compound C concentration to use for HL156 B in further studies. Our results suggest that nuclear FOXM1 expression was inhibited by HL156 (Fig. 5B).

FOXM1 has been reported to crosstalk not only with vascular endothelial growth factor (VEGF) signals, but also with multiple oncogenic pathways, such as phosphatidylinositol 3-kinase (PI3K)/Akt, nuclear factor-κB (NF-κB), sonic hedgehog (Shh), extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK), cyclooxygenase-2 (COX-2), epidermal growth factor receptor (EGFR), estrogen receptor (ER), c-myc, p53, reactive oxygen species (ROS), and hypoxia-inducible factor-1 (HIF-1) signaling [92].

Surprisingly, the analysis from comprehensive molecular evaluation of primary gastric adenocarcinomas as part of The Cancer Genome Atlas (TCGA) project revealed that significantly elevated FOXM1 and PLK1 signaling [93]. Therefore, FOXM1 is an attractive and potential target for anticancer therapeutics [93].

Transcription factors are traditionally thought to be "undruggable" because of transcription factor is a unapproachable nuclear event by therapeutic agents [94]. We have a tool, small molecule inhibitor such as HL156 compound, to target FOXM1, a pleiotropic transcription factor that have multitude of oncogenic effects including angiogenesis, stemness, drug resistance, and EMT.

FOXM1 regulates a variety of biological processes through regulating the transcription of genes important for cell cycle progression, cell proliferation and survival, cell differentiation, DNA damage repair, angiogenesis, cell migration and chemotherapeutic drug response [68]. FOXM1 is required for β-catenin nuclear accumulation in tumor cells, that nuclear FOXM1 and β-catenin form a functional complex with TCF4 on Wnt target-gene promoters, and that the functional interaction between FOXM1 and β-catenin promotes cancer cells self-renewal and tumorigenesis [95]. Thus, it is tempted to target Wnt/β-catenin signaling pathway of which aberrant activation is widespread in human cancers for therapeutic intervention by HL156. We also found that HL156 inhibits migration of gastric cancer cells (Supplementary Fig.1). The role for FOXM1 in the regulation of MMP-2 and MMP-9 expression which related with tumor invasion, metastasis and angiogenesis, is demonstrated in various cancers [96].

From meta-analysis of clinical trials of VEGF inhibitors other than bevacizumab, which include data on 4679 patients from 10 clinical trials, these drugs were associated with fatal adverse events at a rate that was about twice that seen in the placebo groups [97]. There are similar data on bevacizumab, a monoclonal antibody that binds to VEGF. The data on bevacizumab came from a meta-analysis of clinical trials involving 10,217 patients, which found a significantly higher rate of fatal adverse events associated with bevacizumab than with chemotherapy alone. Based on our results, we anticipate that HL156, a new class angiogenesis inhibitor, has a competitive advantage over existing anti-angiogenic drugs in terms of toxicity and efficacy.

Our study suggest that therapeutic targeting of AMPK → CDK4/6 → FOXM1 → VEGFA signaling might be an effective strategy to block angiogenesis in gastric cancer cells, and that small molecule inhibitors should be tested in clinical trials against gastric cancer.

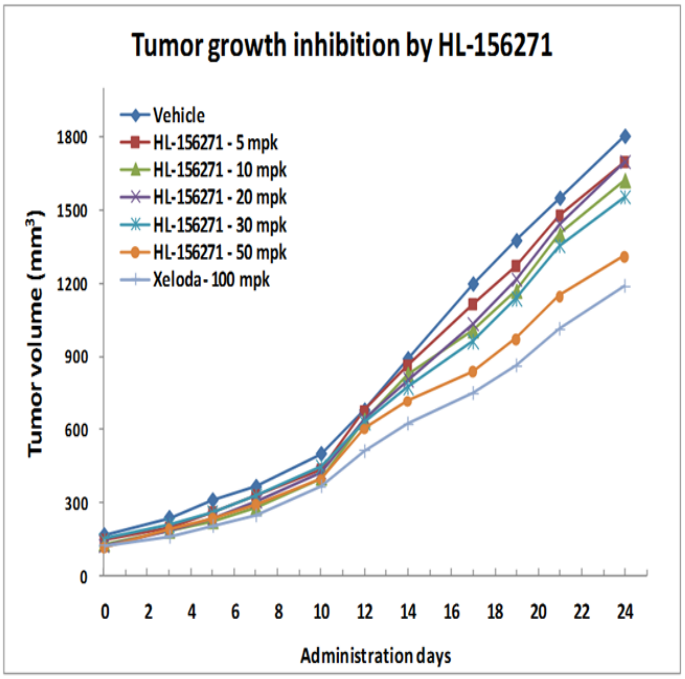
HL156 is a modified compound that is different from metformin with enhanced AMPK activation property. Recently repositioning and repurposing of biguanides to treat cancer is an emerging issue for cancer treatment [98]. HL156 has a documented preclinical safety and efficacy. Therefore, if we establish clinical anti-cancer efficacy of HL156, it will help to treat cancer patients more appropriately.

Ⅴ. CONCLUSION

In conclusion, our data elucidate a novel molecular mechanism of new biguanide compounds in tumor suppression. We demonstrated that HL156 suppressed angiogenesis through down regulating major angiogenic factor VEGFA in human gastric cancer cells. The anti-angiogenic effect was mediated through AMPK activation which reduces FOXM1 by downregulation of CDK4/6. Our findings shed light on the application of AMPK activators in the treatment of human gastric cancer.

Pilot study

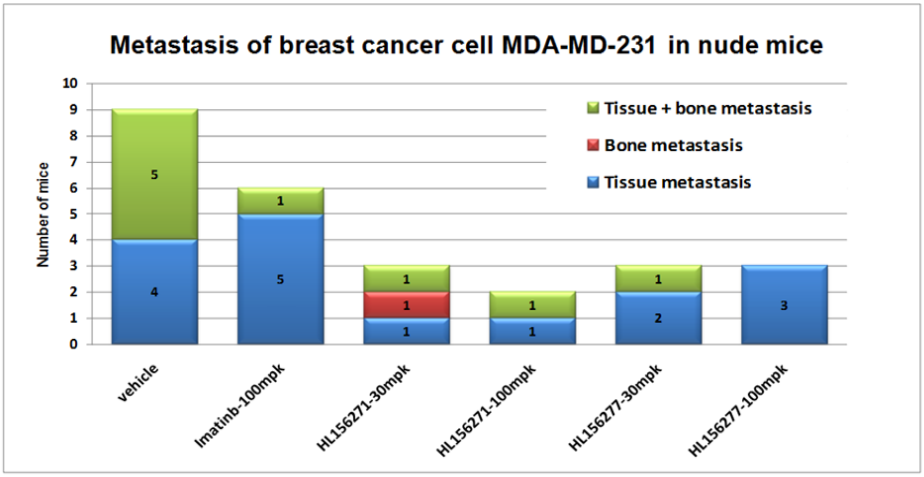
Xenograft model injected human colorectal carcinoma HCT116 cell in nude mice



Group	Tumor Growth Inhibition (%)
Vehicle	0.0
HL-156271 - 5mpk	5.8
HL-156271 - 10 mpk	9.9
HL-156271 - 20 mpk	5.7
HL-156271 - 30 mpk	13.9
HL-156271 - 50 mpk	27.1
Xeloda - 100 mpk	33.9

Pilot Study 1. HL156 compounds inhibit tumor growth *in vivo*

HL156 showed comparable tumor growth inhibition effect at lower dose (50mpk) compared to Xeloda (100mpk) that is an orally-administered chemotherapeutic agent used in the treatment of numerous cancers in xenograft model.

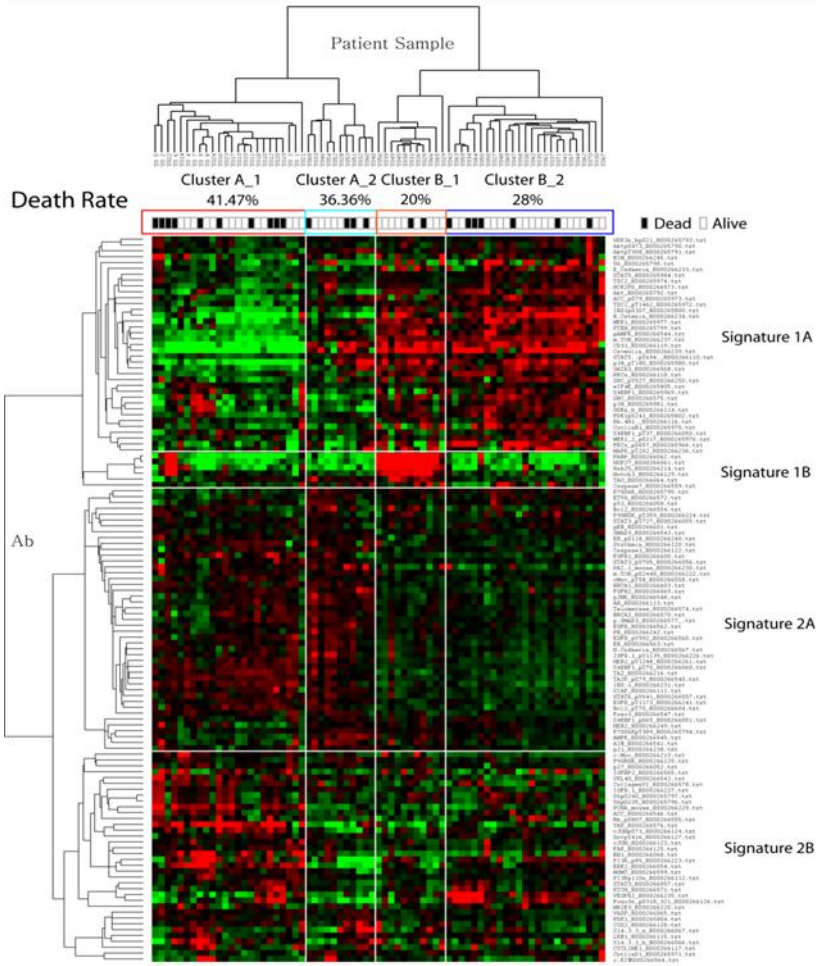


Item	Number of Metastasis					
Test Groups	Vehicle	Imatinib – 100 mpk	**HL156271**-30mpk	**HL156271**-100mpk	**HL156271**-30mpk	**HL15627 7**-100mpk
Bone metastasis	5	1	2	1	1	0
Tissue metastasis	9	6	2	2	3	3
Tissue + Bone metastasis	5	1	1	1	1	0
Total	9	6	3	2	3	3

Pilot study 2. HL156 compounds inhibit tumor metastasis *in vivo*

HL156 showed more than 50% metastasis inhibition effect at lower dose compared to Imatinib (100mpk), marketed by Novartis as Gleevec, is a tyrosine-kinase inhibitor used in the treatment of multiple cancers.

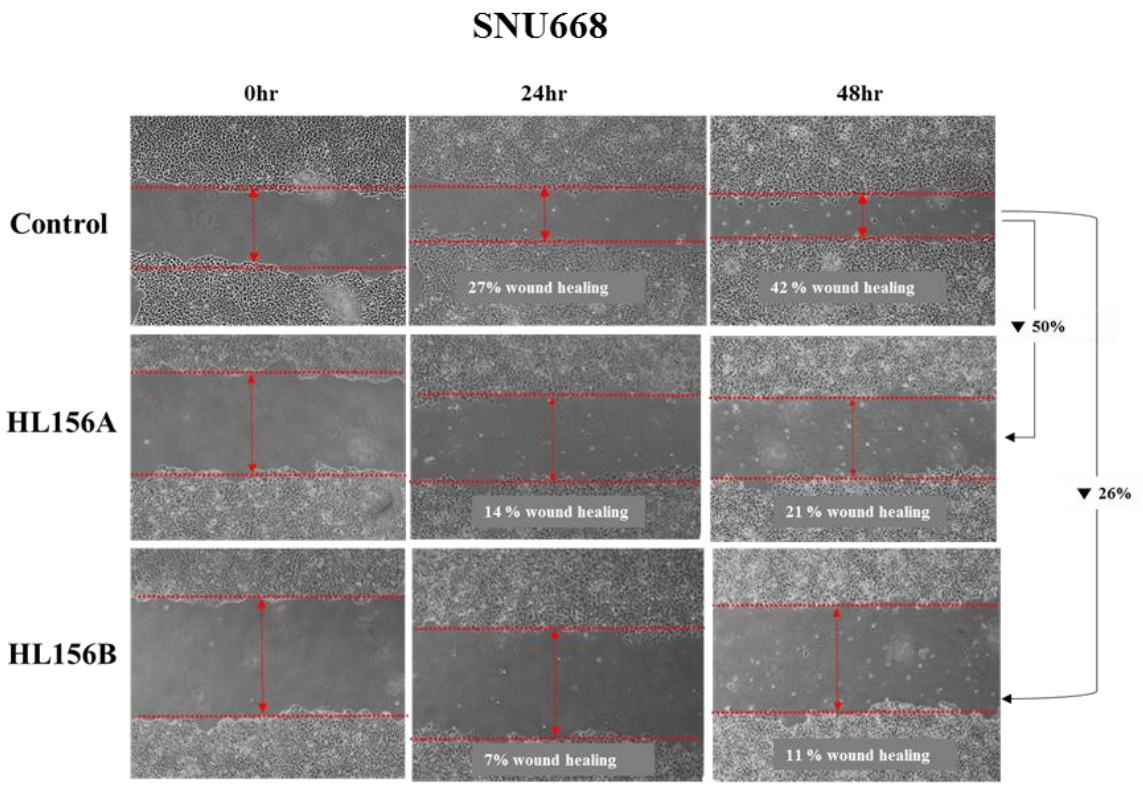
RPPA clustering Analysis (70 Cancer patients, 125 Abs)

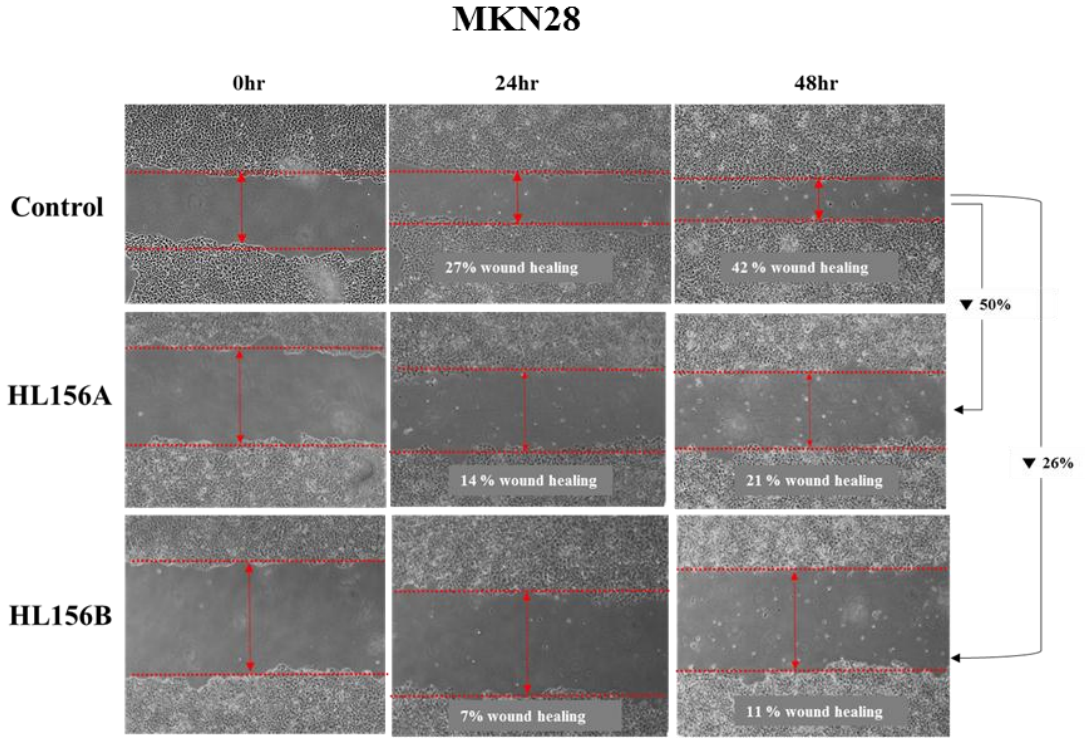


p-value	Hazard Ratio	Unique id
0.019	2.166	**Aktps473**
0.023	1.809	**PAI.1**
0.024	0.165	SMAD3
0.037	0.276	P70S6K
0.049	1.57	**VEGFR2**
0.051	1.52	**Foxo3aps318**

Pilot study 3. Analysis of Reverse phase protein expression array (RPPA) data from 70 gastric cancer patients indicated that p-AKT, PAI1, VEGFR2 and p-FOXO3a which related to angiogenesis increased in tumors of patients with poor prognosis.

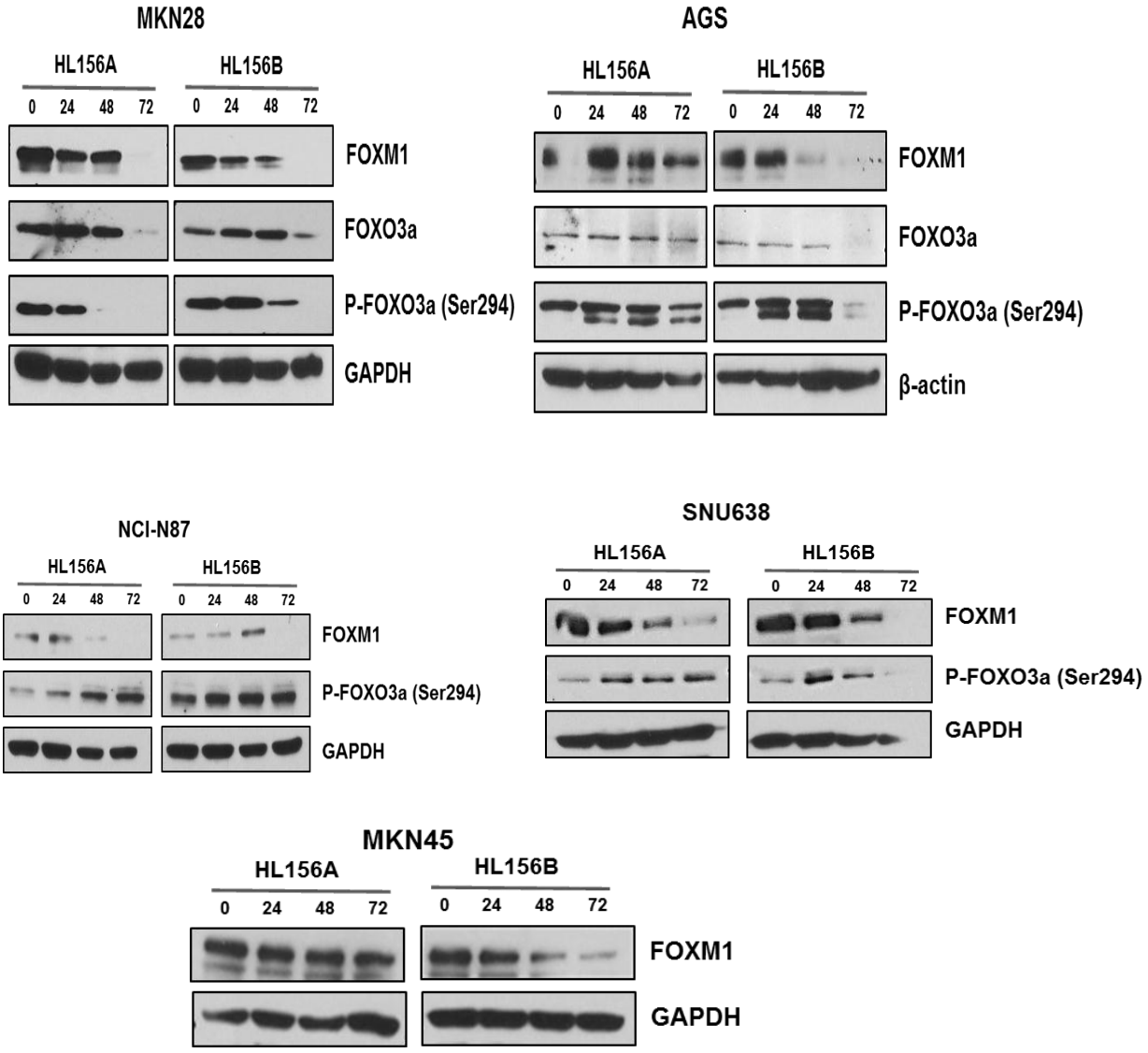
Supplementary data





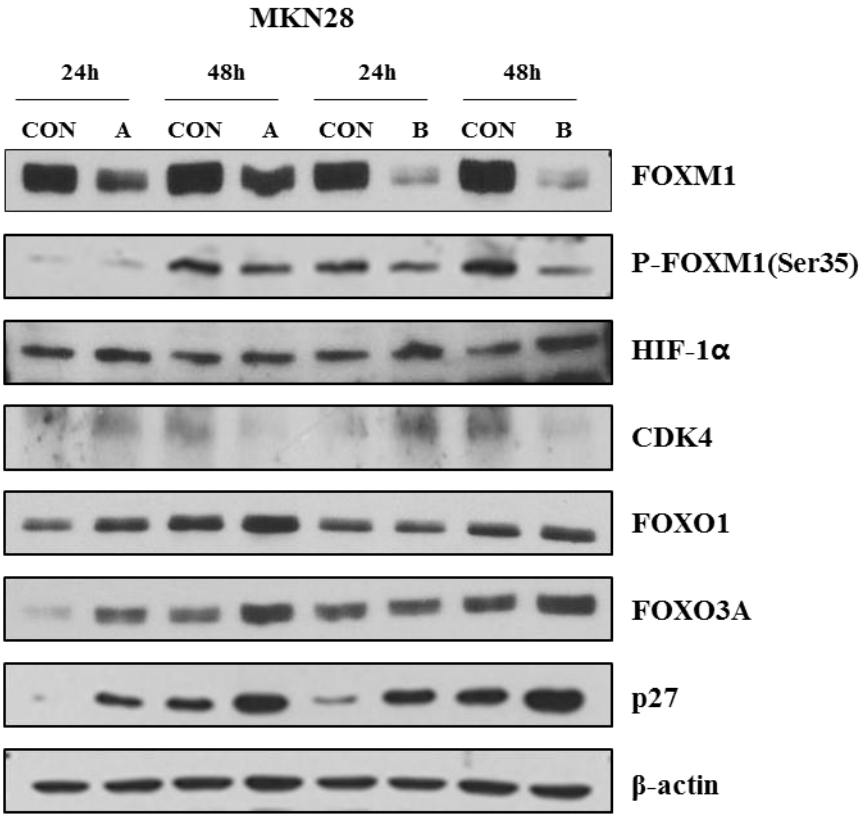
Supplementary Figure 1. HL156 compounds inhibit migration of gastric cancer cells

Wound healing assay for migration was carried out by scratching the cells with yellow tip when gastric cancer cells grew into monolayer. Then, cells incubated with the medium with or without HL156 (30μM) and allowed to migrate into the scratched area for 24 and 48 hours. The arrow showed the gap of scratched area and the percentage of migrating cells in wound healing assay quantified.



Supplementary Figure 2. HL156 compounds repress expression of FOXM1 in several gastric cancer cell lines.

We tested HL156 not only for MKN28 and AGS cells but also for other gastric cancer lineages including NCI-N87, SNU638, and MKN45. We performed immunoblotting with FOXM1 and FOXO3A. The expression of FOXO3A was variable depending on cell lineages, but, expression of FOXM1 distinctly decreased in all of cell lineages. Thus, we assured that FOXM1 is a target of HL156.



Supplementary Figure 3. HL156 influences the expression of various protein levels in MKN28 cells. MKN28, a model cell line, is sensitive to HL156, especially to HL156B. Of note, the results showed that FOXM1, phosphor FOXM1(Ser35), and CDK4 were coordinately down-regulated.

REFERENCES

1. Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. Bmj 2005;330(7503):1304-5.

2. Ben Sahra I, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auberger P, et al. The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. Oncogene 2008;27(25):3576-86.

3. Phoenix KN, Vumbaca F, Claffey KP. Therapeutic metformin/AMPK activation promotes the angiogenic phenotype in the ERalpha negative MDA-MB-435 breast cancer model. Breast cancer research and treatment 2009;113(1):101-11.

4. Zhou XZ, Xue YM, Zhu B, Sha JP. [Effects of metformin on proliferation of human colon carcinoma cell line SW-480]. Nan fang yi ke da xue xue bao = Journal of Southern Medical University 2010;30(8):1935-8, 42.

5. Isakovic A, Harhaji L, Stevanovic D, Markovic Z, Sumarac-Dumanovic M, Starcevic V, et al. Dual antiglioma action of metformin: cell cycle arrest and mitochondria-dependent apoptosis. Cellular and molecular life sciences : CMLS 2007;64(10):1290-302.

6. Kato K, Gong J, Iwama H, Kitanaka A, Tani J, Miyoshi H, et al. The antidiabetic drug metformin inhibits gastric cancer cell proliferation in vitro and in vivo. Molecular cancer therapeutics 2012;11(3):549-60.

7. Hardie DG. AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function. Genes & development 2011;25(18):1895-908.

8. Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M. Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. Cancer research 2006;66(21):10269-73.

9. Pierotti MA, Berrino F, Gariboldi M, Melani C, Mogavero A, Negri T, et al. Targeting metabolism for cancer treatment and prevention: metformin, an old drug with multi-faceted effects. Oncogene 2013;32(12):1475-87.

10. Zhuang Y, Miskimins WK. Cell cycle arrest in Metformin treated breast cancer cells involves activation of AMPK, downregulation of cyclin D1, and requires p27Kip1 or p21Cip1. Journal of molecular signaling 2008;3:18.

11. El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. The Journal of biological chemistry 2000;275(1):223-8.

12. Cheong JH, Park ES, Liang J, Dennison JB, Tsavachidou D, Nguyen-Charles C, et al. Dual inhibition of tumor energy pathway by 2-deoxyglucose and metformin is effective against a broad spectrum of preclinical cancer models. Molecular cancer therapeutics 2011;10(12):2350-62.

13. Tan BK, Adya R, Chen J, Farhatullah S, Heutling D, Mitchell D, et al. Metformin decreases angiogenesis via NF-kappaB and Erk1/2/Erk5 pathways by increasing the antiangiogenic thrombospondin-1. Cardiovascular research 2009;83(3):566-74.

14. Tadakawa M, Takeda T, Li B, Tsuiji K, Yaegashi N. The anti-diabetic drug metformin inhibits vascular endothelial growth factor expression via the mammalian target of rapamycin complex 1/hypoxia-inducible factor-1alpha signaling pathway in ELT-3 cells. Molecular and cellular endocrinology 2014.

15. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature 2008;454(7203):436-44.

16. Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. Oncogene 2008;27(45):5904-12.

17. Poste G, Fidler IJ. The pathogenesis of cancer metastasis. Nature 1980;283(5743):139-46.

18. Fidler IJ. The organ microenvironment and cancer metastasis. Differentiation; research in biological diversity 2002;70(9-10):498-505.

19. Folkman J, Klagsbrun M. Angiogenic factors. Science 1987;235(4787):442-7.

20. Liotta LA. Tumor invasion and metastases--role of the extracellular matrix: Rhoads Memorial Award lecture. Cancer research 1986;46(1):1-7.

21. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA: a cancer journal for

clinicians 2005;55(2):74-108.

22. Fisher B, Fisher ER. The interrelationship of hematogenous and lymphatic tumor cell dissemination. Surgery, gynecology & obstetrics 1966;122(4):791-8.

23. Nicolson GL. Cancer metastasis: tumor cell and host organ properties important in metastasis to specific secondary sites. Biochimica et biophysica acta 1988;948(2):175-224.

24. Liekens S, De Clercq E, Neyts J. Angiogenesis: regulators and clinical applications. Biochemical pharmacology 2001;61(3):253-70.

25. Mignatti P, Rifkin DB. Plasminogen activators and matrix metalloproteinases in angiogenesis. Enzyme & protein 1996;49(1-3):117-37.

26. Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2005;23(5):1011-27.

27. Presta M, Moscatelli D, Joseph-Silverstein J, Rifkin DB. Purification from a human hepatoma cell line of a basic fibroblast growth factor-like molecule that stimulates capillary endothelial cell plasminogen activator production, DNA synthesis, and migration. Molecular and cellular biology 1986;6(11):4060-6.

28. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 1997;277(5322):55-60.

29. Bischoff J. Cell adhesion and angiogenesis. The Journal of clinical investigation 1997;99(3):373-6.

30. Lee JC, Chow NH, Wang ST, Huang SM. Prognostic value of vascular endothelial growth factor expression in colorectal cancer patients. European journal of cancer 2000;36(6):748-53.

31. Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. Cancer research 1995;55(18):3964-8.

32. Takahashi Y, Cleary KR, Mai M, Kitadai Y, Bucana CD, Ellis LM. Significance of vessel count

and vascular endothelial growth factor and its receptor (KDR) in intestinal-type gastric cancer. Clinical cancer research : an official journal of the American Association for Cancer Research 1996;2(10):1679-84.

33. Maeda K, Chung YS, Ogawa Y, Takatsuka S, Kang SM, Ogawa M, et al. Prognostic value of vascular endothelial growth factor expression in gastric carcinoma. Cancer 1996;77(5):858-63.

34. Fujimoto K, Hosotani R, Wada M, Lee JU, Koshiba T, Miyamoto Y, et al. Expression of two angiogenic factors, vascular endothelial growth factor and platelet-derived endothelial cell growth factor in human pancreatic cancer, and its relationship to angiogenesis. European journal of cancer 1998;34(9):1439-47.

35. Ikeda N, Adachi M, Taki T, Huang C, Hashida H, Takabayashi A, et al. Prognostic significance of angiogenesis in human pancreatic cancer. British journal of cancer 1999;79(9-10):1553-63.

36. Berns EM, Klijn JG, Look MP, Grebenchtchikov N, Vossen R, Peters H, et al. Combined vascular endothelial growth factor and TP53 status predicts poor response to tamoxifen therapy in estrogen receptor-positive advanced breast cancer. Clinical cancer research : an official journal of the American Association for Cancer Research 2003;9(4):1253-8.

37. Manders P, Beex LV, Tjan-Heijnen VC, Geurts-Moespot J, Van Tienoven TH, Foekens JA, et al. The prognostic value of vascular endothelial growth factor in 574 node-negative breast cancer patients who did not receive adjuvant systemic therapy. British journal of cancer 2002;87(7):772-8.

38. George DJ, Halabi S, Shepard TF, Vogelzang NJ, Hayes DF, Small EJ, et al. Prognostic significance of plasma vascular endothelial growth factor levels in patients with hormone-refractory prostate cancer treated on Cancer and Leukemia Group B 9480. Clinical cancer research : an official journal of the American Association for Cancer Research 2001;7(7):1932-6.

39. Fontanini G, Lucchi M, Vignati S, Mussi A, Ciardiello F, De Laurentiis M, et al. Angiogenesis as a prognostic indicator of survival in non-small-cell lung carcinoma: a prospective study. Journal of the National Cancer Institute 1997;89(12):881-6.

40. Gorski DH, Leal AD, Goydos JS. Differential expression of vascular endothelial growth factor-A isoforms at different stages of melanoma progression. Journal of the American College of

Surgeons 2003;197(3):408-18.

41. Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2002;20(21):4368-80.

42. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. Nature reviews Molecular cell biology 2007;8(6):464-78.

43. Liu Y, Cox SR, Morita T, Kourembanas S. Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. Circulation research 1995;77(3):638-43.

44. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Molecular and cellular biology 1996;16(9):4604-13.

45. Folkman J. Angiogenesis: an organizing principle for drug discovery? Nature reviews Drug discovery 2007;6(4):273-86.

46. Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. Nature reviews Cancer 2008;8(8):592-603.

47. Grothey A, Sugrue MM, Purdie DM, Dong W, Sargent D, Hedrick E, et al. Bevacizumab beyond first progression is associated with prolonged overall survival in metastatic colorectal cancer: results from a large observational cohort study (BRiTE). Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2008;26(33):5326-34.

48. Gerber HP, Dixit V, Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. The Journal of biological chemistry 1998;273(21):13313-6.

49. Fujio Y, Walsh K. Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. The Journal of biological chemistry 1999;274(23):16349-54.

50. Yao R, Cooper GM. Requirement for phosphatidylinositol-3 kinase in the prevention of

apoptosis by nerve growth factor. Science 1995;267(5206):2003-6.

51. Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, et al. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell 1995;81(5):727-36.

52. Liu L, Zhu S, Gong Z, Low BC. K-ras/PI3K-Akt signaling is essential for zebrafish hematopoiesis and angiogenesis. PloS one 2008;3(8):e2850.

53. Jiang BH, Zheng JZ, Aoki M, Vogt PK. Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. Proceedings of the National Academy of Sciences of the United States of America 2000;97(4):1749-53.

54. Emerling BM, Weinberg F, Liu JL, Mak TW, Chandel NS. PTEN regulates p300-dependent hypoxia-inducible factor 1 transcriptional activity through Forkhead transcription factor 3a (FOXO3a). Proceedings of the National Academy of Sciences of the United States of America 2008;105(7):2622-7.

55. Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nature reviews Genetics 2006;7(8):606-19.

56. Potente M, Urbich C, Sasaki K, Hofmann WK, Heeschen C, Aicher A, et al. Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. The Journal of clinical investigation 2005;115(9):2382-92.

57. Daly C, Wong V, Burova E, Wei Y, Zabski S, Griffiths J, et al. Angiopoietin-1 modulates endothelial cell function and gene expression via the transcription factor FKHR (FOXO1). Genes & development 2004;18(9):1060-71.

58. Potente M, Fisslthaler B, Busse R, Fleming I. 11,12-Epoxyeicosatrienoic acid-induced inhibition of FOXO factors promotes endothelial proliferation by down-regulating p27Kip1. The Journal of biological chemistry 2003;278(32):29619-25.

59. Karadedou CT, Gomes AR, Chen J, Petkovic M, Ho KK, Zwolinska AK, et al. FOXO3a represses VEGF expression through FOXM1-dependent and -independent mechanisms in breast cancer. Oncogene 2012;31(14):1845-58.

60. Laoukili J, Kooistra MR, Bras A, Kauw J, Kerkhoven RM, Morrison A, et al. FoxM1 is required for execution of the mitotic programme and chromosome stability. Nature cell biology 2005;7(2):126-36.

61. Laoukili J, Stahl M, Medema RH. FoxM1: at the crossroads of ageing and cancer. Biochimica et biophysica acta 2007;1775(1):92-102.

62. Korver W, Roose J, Clevers H. The winged-helix transcription factor Trident is expressed in cycling cells. Nucleic acids research 1997;25(9):1715-9.

63. Ye H, Kelly TF, Samadani U, Lim L, Rubio S, Overdier DG, et al. Hepatocyte nuclear factor 3/fork head homolog 11 is expressed in proliferating epithelial and mesenchymal cells of embryonic and adult tissues. Molecular and cellular biology 1997;17(3):1626-41.

64. Yao KM, Sha M, Lu Z, Wong GG. Molecular analysis of a novel winged helix protein, WIN. Expression pattern, DNA binding property, and alternative splicing within the DNA binding domain. The Journal of biological chemistry 1997;272(32):19827-36.

65. Luscher-Firzlaff JM, Westendorf JM, Zwicker J, Burkhardt H, Henriksson M, Muller R, et al. Interaction of the fork head domain transcription factor MPP2 with the human papilloma virus 16 E7 protein: enhancement of transformation and transactivation. Oncogene 1999;18(41):5620-30.

66. Korver W, Roose J, Heinen K, Weghuis DO, de Bruijn D, van Kessel AG, et al. The human TRIDENT/HFH-11/FKHL16 gene: structure, localization, and promoter characterization. Genomics 1997;46(3):435-42.

67. Myatt SS, Lam EW. The emerging roles of forkhead box (Fox) proteins in cancer. Nature reviews Cancer 2007;7(11):847-59.

68. Koo CY, Muir KW, Lam EW. FOXM1: From cancer initiation to progression and treatment. Biochimica et biophysica acta 2012;1819(1):28-37.

69. Park HJ, Gusarova G, Wang Z, Carr JR, Li J, Kim KH, et al. Deregulation of FoxM1b leads to tumour metastasis. EMBO molecular medicine 2011;3(1):21-34.

70. Wierstra I, Alves J. Transcription factor FOXM1c is repressed by RB and activated by cyclin D1/Cdk4. Biological chemistry 2006;387(7):949-62.

71. Anders L, Ke N, Hydbring P, Choi YJ, Widlund HR, Chick JM, et al. A systematic screen for CDK4/6 substrates links FOXM1 phosphorylation to senescence suppression in cancer cells. Cancer cell 2011;20(5):620-34.

72. Ma RY, Tong TH, Cheung AM, Tsang AC, Leung WY, Yao KM. Raf/MEK/MAPK signaling stimulates the nuclear translocation and transactivating activity of FOXM1c. Journal of cell science 2005;118(Pt 4):795-806.

73. Choi YJ, Anders L. Signaling through cyclin D-dependent kinases. Oncogene 2014;33(15):1890-903.

74. Smith JK, McPhee JT, Hill JS, Whalen GF, Sullivan ME, Litwin DE, et al. National outcomes after gastric resection for neoplasm. Archives of surgery 2007;142(4):387-93.

75. Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. Nature reviews Drug discovery 2005;4(12):988-1004.

76. Cinti C, Vindigni C, Zamparelli A, La Sala D, Epistolato MC, Marrelli D, et al. Activated Akt as an indicator of prognosis in gastric cancer. Virchows Archiv : an international journal of pathology 2008;453(5):449-55.

77. Jeon TY, Han ME, Lee YW, Lee YS, Kim GH, Song GA, et al. Overexpression of stathmin1 in the diffuse type of gastric cancer and its roles in proliferation and migration of gastric cancer cells. British journal of cancer 2010;102(4):710-8.

78. Dallaglio K, Bruno A, Cantelmo AR, Esposito AI, Ruggiero L, Orecchioni S, et al. Paradoxic effects of metformin on endothelial cells and angiogenesis. Carcinogenesis 2014;35(5):1055-66.

79. Xavier DO, Amaral LS, Gomes MA, Rocha MA, Campos PR, Cota BD, et al. Metformin inhibits inflammatory angiogenesis in a murine sponge model. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 2010;64(3):220-5.

80. Lin C, McGough R, Aswad B, Block JA, Terek R. Hypoxia induces HIF-1alpha and VEGF expression in chondrosarcoma cells and chondrocytes. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 2004;22(6):1175-81.

81. Wang IC, Chen YJ, Hughes DE, Ackerson T, Major ML, Kalinichenko VV, et al. FoxM1

regulates transcription of JNK1 to promote the G1/S transition and tumor cell invasiveness. The Journal of biological chemistry 2008;283(30):20770-8.

82. Wang Z, Banerjee S, Kong D, Li Y, Sarkar FH. Down-regulation of Forkhead Box M1 transcription factor leads to the inhibition of invasion and angiogenesis of pancreatic cancer cells. Cancer research 2007;67(17):8293-300.

83. Zhang Y, Zhang N, Dai B, Liu M, Sawaya R, Xie K, et al. FoxM1B transcriptionally regulates vascular endothelial growth factor expression and promotes the angiogenesis and growth of glioma cells. Cancer research 2008;68(21):8733-42.

84. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. The Journal of clinical investigation 2001;108(8):1167-74.

85. Hardie DG. Neither LKB1 nor AMPK are the direct targets of metformin. Gastroenterology 2006;131(3):973; author reply 4-5.

86. Mihaylova MM, Shaw RJ. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. Nature cell biology 2011;13(9):1016-23.

87. Sengupta A, Kalinichenko VV, Yutzey KE. FoxO1 and FoxM1 transcription factors have antagonistic functions in neonatal cardiomyocyte cell-cycle withdrawal and IGF1 gene regulation. Circulation research 2013;112(2):267-77.

88. Yung MM, Chan DW, Liu VW, Yao KM, Ngan HY. Activation of AMPK inhibits cervical cancer cell growth through AKT/FOXO3a/FOXM1 signaling cascade. BMC cancer 2013;13:327.

89. Halasi M, Gartel AL. Targeting FOXM1 in cancer. Biochemical pharmacology 2013;85(5):644-52.

90. Xue YJ, Xiao RH, Long DZ, Zou XF, Wang XN, Zhang GX, et al. Overexpression of FoxM1 is associated with tumor progression in patients with clear cell renal cell carcinoma. Journal of translational medicine 2012;10:200.

91. Ben Sahra I, Le Marchand-Brustel Y, Tanti JF, Bost F. Metformin in cancer therapy: a new perspective for an old antidiabetic drug? Molecular cancer therapeutics 2010;9(5):1092-9.

92. Wang Z, Ahmad A, Li Y, Banerjee S, Kong D, Sarkar FH. Forkhead box M1 transcription

factor: a novel target for cancer therapy. Cancer treatment reviews 2010;36(2):151-6.

93. Cancer Genome Atlas Research N. Comprehensive molecular characterization of urothelial bladder carcinoma. Nature 2014;507(7492):315-22.

94. Yan C, Higgins PJ. Drugging the undruggable: transcription therapy for cancer. Biochimica et biophysica acta 2013;1835(1):76-85.

95. Zhang N, Wei P, Gong A, Chiu WT, Lee HT, Colman H, et al. FoxM1 promotes beta-catenin nuclear localization and controls Wnt target-gene expression and glioma tumorigenesis. Cancer cell 2011;20(4):427-42.

96. Ahmad A, Wang Z, Kong D, Ali S, Li Y, Banerjee S, et al. FoxM1 down-regulation leads to inhibition of proliferation, migration and invasion of breast cancer cells through the modulation of extra-cellular matrix degrading factors. Breast cancer research and treatment 2010;122(2):337-46.

97. Schutz FA, Je Y, Richards CJ, Choueiri TK. Meta-analysis of randomized controlled trials for the incidence and risk of treatment-related mortality in patients with cancer treated with vascular endothelial growth factor tyrosine kinase inhibitors. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2012;30(8):871-7.

98. Pollak M. Overcoming Drug Development Bottlenecks With Repurposing: Repurposing biguanides to target energy metabolism for cancer treatment. Nature medicine 2014;20(6):591-3.

ABSTRACT (IN KOREA)

위암 세포에서 새로운 메트포르민 유도체 HL156 화합물의 AMPK 경유 FOXM1 억제에 의한 항신생혈관 효과

<지도교수 정재호>

연세대학교 대학원 의과학과

최혜지

메트포르민은 널리 사용되고 있는 바이구아니드계 경구형 당뇨 치료제로서 최근에 항암제로서의 가능성이 제시되고 있다. 하지만 메트포르민의 항암효과의 작용기전은 분자적 수준에서 잘 밝혀져 있지 않다. HL156 화합물은 메트포르민 분자식을 바탕으로 개발된 바이구아니드계 유도체로서 다양한 암세포주 대상 선행 실험 결과에서 메트포르민보다 증가된 AMPK 활성 효과 및 더 뛰어난 항암효과를 보여주었다.

선행연구로 수행된 위암환자 종양조직의 단백체 분석 (reverse phase protein array, RPPA) 결과 신생혈관 관련 단백질 발현과 환자 예후 사이에 유의한 상관 관계가 있음을 확인하였다. 또한 마우스 종양 모델에서 HL156 화합물의 전이 억제효과에 기반하여, 저자는 위암세포모델에서 암전이의 필수단계인 혈관신생 억제에 대한 HL156 화합물의 효과를 규명하기 위한 연구를 수행하였다. 위암세포에 HL156 화합물을 처리해서 얻은 조건화 배지를 이용하여 시험관상에서 혈관 내피 세포의 신생혈관이 억제되는 것을 확인하였다. 또한 조건화 배지에서 주요 신생혈관 유도인자인 VEGFA 의 발현 및 분비량이 유의하게 감소하는 것을 ELISA 를 통해 확인하였다. 나아가 HL156 처리시 위암세포에서 AMPK 활성을 통해 VEGFA 유전자 발현을 조절하는 종양형성전사인자인 FOXM1 발현이 감소하는 것을 확인하였다. 이러한 효과는 FOXM1 을 인산화 시키는 CDK4/6 키나제가 발현 억제되어 생물학적으로 안정된 형태의 phosho-FOXM1 발현 수준 저하와 상관되어있음을 확인 하였다. 마지막으로 electrophoretic mobility shift assay (EMSA) 를 통해 HL156 처리시 FOXM1 의 VEGFA 프로모터 결합이 억제됨을 확인하였다. 결론적으로 본 연구 결과는 HL156 화합물에 의한 세포내 AMPK 활성이 CDK4/6 발현을 억제하여 종양형성전사인자인 FOXM1 의 탈인산화 및 핵내 발현을 감소시키므로서 표적 유전자인 VEGFA 발현 억제를 통해 혈관신생을 억제하는 새로운 분자 기전을 제시한다.

핵심되는 말 : HL156, 신생혈관 , gastric cancer, ampk, foxm1, cdk4/6